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Pollutants associated with mass mortality of Nile crocodiles (*Crocodylus niloticus*) in the Kruger National Park, South Africa.

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List of abbreviations

AMD	Acid mine drainage
asl	Above sea level
ATSDR	Agency for Toxic Substances and Disease Registry
BFR	Brominated flame retardants
BPA	Bisphenol A
CNS	Central nervous system
CROC	Consortium for the Restoration of the Olifants Catchment
DDT	Dichlorodiphenyl trichloroethane
DNA	Deoxyribonucleic acid
EDC	Endocrine disrupting chemical
GPS	Global Positioning System
HDPE	High density polyethylene
HR-GC	High – resolution gas chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
IWMI	International Water Management Institute
KNP	Kruger National Park
LOQ	Level of quantification
MRPP	Multi-response permutation procedure
NVH	Norwegian School for Veterinarian Science
PCA	Principle component analysis

PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzodioxins
PCDF	Polychlorinated dibenzofurans
POPs	Persistent organic pollutants
PVC	Polyvinyl chloride
RHP	River Health Program
RNA	Ribonucleic acid
SA	South Africa
SANParks	South African National Parks
SAPS	South African Police Service
SEM	Scanning electron microscope
TSD	Temperature – dependant sex determination
ULP	Ultrasonic liquid processor
USEPA	United States Environmental Protection Agency
WISA	Water Institute of Southern Africa
WM	Wet mass

Abstract

Pollutants associated with mass mortality of Nile crocodiles (*Crocodylus niloticus*) in the Kruger National Park, South Africa.

The first of a series of mass mortalities of Nile crocodiles in the Olifants and Letaba rivers in the Kruger National Park (KNP) was reported in the winter of 2008. The present study investigated the levels and possible effects on eggshell thickness of inorganic elements and organic pollutants in Nile crocodile eggs from these rivers, and comparing them with eggs from a reference crocodile farm and a reference dam inside the KNP.

The egg contents were analyzed for chlorinated organic compounds and brominated flame retardants. Eggshells and egg contents were analyzed for inorganic elements.

The elemental concentrations in the eggshells and contents were low when compared with previous studies. The highest concentrations were found in the eggs from the reference crocodile farm. The eggs from the reference dam and the crocodile farm had thicker shells, and the eggs from the Olifants and Letaba rivers had thinner shells.

Not all eggs in a female develop at the same rate, while eggshell formation presumably occurs at the same time for all eggs. As a result, the elemental profile of egg contents may differ between eggs of the same clutch, but less so for the shells. Weak or no associations were found between the elemental concentrations of the content and eggshells and eggshell thinning. A possible organic pollutant-induced eggshell thinning effect was found.

The compounds found were not at levels that could have caused the mortalities, but may affect the sex ratios through endocrine disruption. Further studies are therefore required.

Key words: Kruger National Park, crocodile farm, Olifants River, Letaba River, Nhlanganini Dam, chlorinated organic compounds, brominated flame retardants, inorganic elements, eggshell thinning.

Opsomming

Besoedelstowwe geassosieer met die massa sterfte van Nyl krokodille (*Crocodylus niloticus*) in die Nasionale Kruger Wildtuin, Suid-Afrika.

Die massasterftes van Nyl krokodille is die eerste keer opgemerk in die winter van 2008 in die Olifantsrivier en Letabarivier in die Nasionale Kruger Wildtuin (NKW). Hierdie studie het ondersoek ingestel na die vlakke en moontlike effekte op eierdopdikte van anorganiese elemente en organiese besoedelstowwe in krokodileiers, en het dit met vlakke in krokodileiers van ‘n krokodilplaas en ‘n dam in die NKW as verwysings vergelyk.

Die eierinhoud is geanaliseer vir gechloreerde besoedelstowwe en gebromineerde vlamonderdrukkers, en die inhoud en doppe vir anorganiese elemente.

Die elemente in die inhoud en eierdoppe was laag gewees in vergelyking met die konsentrasies in die inhoud en eierdoppe in die literatuur. Die hoogste elementkonsentrasies was in die eiers vanaf die krokodilplaas. Die eiers van die verwysingsdam en krokodilplaas het die dikste doppe gehad, en die eiers van die Letaba- en Olifantsriviere die dunste.

Nie alle eiers in die wyfie ontwikkel teen dieselfde koers nie, maar dopneerlegging gebeur waarskynlik terselfdertyd in alle eiers. Dit mag verklaar hoekom die elementprofiel in die eiers van dieselfde nes minder ooreenstem as die van die eierdoppe. Swak of geen assosiasie was gevind tussen eierdopdiktes en elementvlakke van die eierinhoud of doppe nie. ‘n Moontlike assosiasie tussen die organiese besoedelstowwe en eierdopdikte is waargeneem.

Alhoewel die konsentrasie van elemente en besoedelstowwe die geslagsverhouding van die nes kan affekteer, was dit nie hoog genoeg om mortaliteite te kon veroorsaak nie. Verdere ondersoeke is dus aangewys.

Sleutelwoorde: Nasionale Kruger Wildtuin, Olifants Rivier, Letaba Rivier, Nhlanganini Dam, gechloreerde organiese verbindings, gebromineerde vlamonderdrukkers, anorganiese elemente, eierdopverdunning.

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1. Introduction

1.1. Background

The Massingir Dam was built on the Mozambique side of the South African border in the 1970s. After cessation of the civil war, the Mozambican Government decided to raise the dam walls which would allow the dam to function at a higher capacity, eventually holding 2 800 million m^3 water. The construction started in 2004 and completion was planned for 2006. However, complications extended construction into 2008. A spokesperson for the Kruger National Park (KNP), Raymond Travers, voiced concern that the raising of the dam walls would cause sediment to push up into the Olifants Gorge (SANParks Forum, 2005).

On 27 May 2008, a bloated dead crocodile was spotted at the confluence of the Olifants and Letaba rivers. This led to a thorough search of the entire river system inside the KNP. More crocodile carcasses were found, with the total body count at the end of November 2008 at 170.

Autopsies on the dead crocodiles by veterinarians confirmed that the deaths were mediated by pansteatitis, but the cause could not be found. Also known as yellow-fat-disease, pansteatitis has been found in animals such as rainbow trout (*Oncorhynchus mykiss*; Roberts *et al.*, 2006), white sturgeon (*Acipenser transmontanus*; Guarda *et al.*, 1997), Atlantic halibut (*Hippoglossus hippoglossus L.*; Bricknell *et al.*, 1996), northern bluefin tuna (*Thunnus thynnus L.*; Roberts & Agius, 2008), red tailed hawk (*Buteo jamaicensis*; Wong *et al.*, 1999), boat billed herons (*Cochlearius cochlearius*; Pollock *et al.*, 1999), the domestic cat (*Felis catus*; Niza *et al.*, 2003), wild rabbit (Jones *et al.*, 1969), marmoset (*Callithix* spp.; Juan-Sallés *et al.*, 2003), and the Amazon River dolphin (*Inia geoffrensis*; Bonar & Wagner, 2003). Pansteatitis is a disease commonly associated with depletion in vitamin E, and is characterized by inflammation and colour change of the fat (Osthof *et al.*, 2010). The depletion of vitamin E is often brought about by a diet of unsaturated fatty acids (Osthof *et al.*, 2010). In the case of the KNP crocodiles, the source of unsaturated fatty acids was suspected to be dead fish, although no fish deaths were recorded during that time.

Further studies were conducted by a number of scientists from the private sector, some South-African universities, South African Police Service's (SAPS) forensics, and the Scientific Services of SANParks. Water, sediment, invertebrates, fish and crocodile samples were taken for analysis by the North-West University (NWU). At the end of 2008, the Consortium for the Restoration of the Olifants Catchment (CROC) was established by scientists from different fields in order to investigate and manage the problem as a matter of urgency. The winter of 2009 saw another series of crocodile deaths in the same area, but with deaths also in the Sabie River, under similar circumstances as in the Olifants and Letaba rivers. In 2010, as mortalities continued, autopsies were done on fish from the Olifants and Letaba rivers and more samples were taken for further tests.

1.2. The Olifants River

1.2.1. General background

The Olifants Catchment originates in the Highveld grasslands, covers 54 570 km^2 and flows into the Massingir Dam in Mozambique, after passing through Gauteng, Mpumalanga, Limpopo Province and the KNP (IWMI, 2007). The river's main tributaries are the Klein Olifants River, Elands River, Wilge River, and Bronkhorstspruit River (RHP, 2001). The dams associated with the system may be as many as 2 500, with 30 of them being major dams (IWMI, 2007). Some of the major dams are the Loskop Dam, Witbank Dam, Middelburg Dam, Blyderivierspoort Dam and many others (RHP, 2001).

According to the South African River Health Programme (RHP, 2001), the mean annual runoff in the system is 2 400 million m^3. This estimation is in contrast with the 1 992 million m^3 estimated by the International Water Management Institute (IWMI, 2007). In 2007 this was still more than the estimated 976 million m^3 water demand, although in 2010 the demand was calculated to be 1 210 million m^3.

Both the RHP (2001) and the IWMI (2007) reports state that the main land-uses around the Olifants Catchment are agriculture (approximately 100 000 ha), forestry (approximately 71 500 ha), conservation (approximately 20 000 ha) and mining. These activities place the system under tremendous pressure. In areas dominated by agriculture, overgrazing and heavy erosion of the banks of the river impacts in such a way that the river turns red after heavy rains (RHP, 2001). The water from the Olifants Catchment is mostly used directly from the river for irrigation and mining uses, although the dams are also used for recreation and conservation. The 3.4 million inhabitants of the Olifants Catchment can be divided into two groups: one group with neither sanitation nor modern water supply systems, and the other with both.

The Olifants River and its tributaries, impoundments, and wetlands support a great number of aquatic and terrestrial vertebrates and invertebrates. The system is home to a number of endemic fish species and frogs (WISA, 2006). The Olifants, and its tributaries, thus play a very important role in maintaining life, and bear a huge socio-economic value.

1.2.2. Eco-regions and climate

Because of the vastness of the catchment it can be expected that the catchment would fall within regions differing in geomorphology, rainfall, and vegetation types. Topographically, the river descends from 2 300 m to 300 m above sea level (asl) in South Africa (SA). The entire system falls within the summer rainfall area of South Africa (RHP, 2001). Rainfall within the Olifants Catchment area can differ between 400 - 1 500 mm annually, with an annual mean of 631 mm. The mean annual temperatures vary between 10 - 22°C. The area can be divided into seven eco-regions, each with its own sub-regions: Plains, Central Highlands, Bushveld Basin, Great Escarpment Mountain, Lowveld, Lebombo Uplands and Highveld (RHP, 2001). The geology of the catchment is varied as it consists of quartzite, sandstone, carbonaceous shale, andesite, conglomerate, basalt, syenite, hornblende granite, and coal (RHP, 2001). The main vegetation types range from North-eastern Mountain Grassland to Afromontane Forest patches. Other vegetation types are Mixed Bushveld, Mopane Bushveld and Shrubveld, Sour -and Sweet Lowveld Bushveld, Lebombo Arid Mountain Bushveld to name but a few (RHP, 2001).

1.2.3. Activities in the Olifants Catchment

Mining, agriculture, and rural development are the major activities in the Olifants Catchment (IWMI, 2007). The most common mining are for coal, which is also the second largest mining industry in South Africa (Hobbs *et al.*, 2008). The ecology of the upper parts of the Olifants River is under tremendous stress due to acid mine drainage (AMD) caused by a lack of proper management (Hobbs *et al.*, 2008). Van Zyl *et al.* (2001), reports that the river is polluted with high concentrations of metals and elevated sulphate levels. Hobbs *et al.* (2008), reports that the Witbank, Highveld, and Ermelo coalfields produce coal for power generation equal to 48% of South Africa's total generating capacity. Some of the process water ends up in the upper Olifants Catchment. Van Zyl *et al.* (2001) calculated that the mine water in the Olifants River at that time (2001) contributed only 4.6% of the total water, but contributed 78.4% of the sulphate load in the system. The 100 mg/ℓ threshold for aquatic ecosystem health and the 200 mg/ℓ threshold for sulphate levels for human consumption have been exceeded since 2001 (de Villiers & Mkwelo, 2009). Without any mining activities in the upper Olifants region, the estimated sulphate concentrations would be as low as 20 – 40 mg/ℓ (van Zyl *et al.*, 2001). De Villiers and Mkwelo (2009) blames bad management, lack of frequent monitoring, and the deposition of sulphate rich water in the catchment as the primary reasons for the toxicity of the water.

Apart from the mining industry, agriculture is also adding stress on the ecological functioning of the Olifants River. Irrigation farming is partially responsible for the salinization and increased levels of chlorine in the Olifants River's Loskop Valley (Aihoon *et al.,* 1997). The agricultural society in the Olifants River catchment ranges between sophisticated farmers with high-value crops to small-scale farmers (IWMI, 2007). In an environmental framework report on the Olifants and Letaba rivers, the condition of the upper reaches of the Olifants (from the source to the confluence with the Steenkoolspruit) is described as relatively good with little impact. This is ascribed to dry-land agriculture being the main land-use. From there, on to the confluence with the Wilge River, the condition of the river is described as poor and very poor, due to coal mining. From Bronkhorstspruit Dam to Premier Dam the influence of irrigation farming is dominant, with river water also in poor condition. The worst water quality in the upper

Olifants River is found between the Arabie Dam and downstream of the Mohlapitse confluence. This area is in very poor condition due to irrigation return flows, poor land-use practices, suspended sediment loads, and evaporation losses concentrating salts in the water. From the Mohlapitse confluence to the area of the Steelpoort River, the condition remains very poor, until it is improved by the clean water from the Blyde River (MetroGIS, 2010). At present, the Olifants River is one of South Africa's most threatened river systems (de Villiers & Mkwelo, 2009).

With a large diversity of fauna and flora depending on the Olifants River Catchment, the current situation poses a serious threat. In *Oreochromis mossambicus* and *Clarias gariepinus* from the Olifants River*,* high concentrations of copper and zinc were found in the muscle, skin, gills, and liver (Kotze *et al.*, 1999). The possibility of accumulation of other metals in various aquatic species found in the Olifants River was intensively investigated during the last two decades. Different metals are accumulated by different organs in different concentrations, depending on the function of the organ and the availability of the metal (du Preez & Steyn, 1992; Avenant-Oldewage & Marx, 2000; Seymore *et al.*, 1995; Robinson & Avenant-Oldewage, 1997; Grobler *et al.*, 1994). Recent studies have indicated that the raising of the Loskop Dam wall in 1979 resulted in a decline in Nile crocodile populations. The rising water levels caused the distribution of the crocodiles to shift into areas with higher pollution concentrations. Mass die-offs (similar to those currently in the KNP) were recorded since 2005 (Botha *et al.,* 2010).

Further rural development and the ongoing use of DDT in these areas for malaria control is another stress factor adding to the already degraded condition of the Olifants River (Grobler, 1994) due to its adverse effects on biota (Yu, 2005). In 1994, DDT and other organic pollutants were found to be present in the sediment, water, and various fish species in the lower reaches of the Olifants River inside the borders of the KNP (Grobler, 1994).

1.3 The Letaba River

1.3.1. General background

The Letaba River Catchment covers 13 670 km^2 originating in the Drakensberg, and joins the Olifants River inside the KNP (RHP, 2001a). This perennial river runs through steep cliffs, occasionally forming waterfalls, passing a variety of eco-regions and forming many deep pools. The Letaba Catchment has a mean annual precipitation of 612 mm, of which more than 60% derives from only 6% of the catchment (RHP, 2001a). This implies that the greater part of the catchment runs through dry regions. Mean annual runoff is estimated at 574 million m^3 of which 10% is from the mean annual precipitation in the wetter regions, to less than 2% in the drier regions (RHP, 2001a). The flow of the rivers in the catchment is impeded by more than 20 dams, which include major constructions such as the Tzaneen and the Middle Letaba dams (RHP, 2001a).

1.3.2. Eco-regions and climate

Twelve eco-regions characterize the Letaba Catchment, varying from Mopane Bushveld to North-eastern Mountain Grassland with some patches of Afromontane Forest (RHP, 2001a). As a result of the elevation differences (2 100 – 200 m asl) the temperatures of the catchment range between -8°C - 46°C (RHP, 2001a) during the year. The mean annual rainfall ranges between 200 - 2 025 mm. A mixture of soils from sandy to gravel, characterizes the bottom of the river. The geology of the Letaba Catchment consists of sandstone, quartzite, shale, granite, conglomerate, and basalt (to name a few) (RHP, 2001a).

1.3.3. Activities in the Letaba catchment

1 130 000 people inhabit the Letaba Catchment, with only a few of them in formal towns. Only 6% of these people have access to reticulated water (RHP, 2001a). A large part of the catchment falls within the borders of the KNP, which has a very low population density. A number of areas of importance are found across the catchment. This includes the Wolkberg Wilderness area (which is considered a biodiversity

hotspot), the Modjadji Cycad Reserve, Lake Fundudzi, and many other areas of economic and environmental importance (RHP, 2001a).

Tourism, forestry, agriculture, infrastructure, and the introduction of exotic fish and plants have come to play a major role in the functionality of the system (RHP, 2001a). Apart from plantations, numerous alien plants (such as the caster-oil, *Sesbenia*, and wild tobacco) have decreased the availability of water for the natural fauna and flora (RHP, 2001a). Further decreased water availability is brought about by irrigation farming (RHP, 2001a). Floods are an annual occurrence in the Letaba Catchment. Siltation is common in the winter months, which is brought on by bad agricultural practice, poor management, construction, and poor environmental knowledge. Pesticides and fertilizers increase the stress in the catchment (RHP, 2001a). In the KNP, the Letaba River experiences only ephemeral flows (Moon & Heritage, 2001). The general condition of the Letaba catchment is fair to poor, although the condition inside the KNP is described as good. The Letaba River is much less polluted than the Olifants River; the worst threat is the decline in flow (Vlok & Engelbrecht, 2000). Constructions such as dams and bridges block natural migration routes of indigenous fish such as tiger fish (*Hydrocynus vittatus*) and the large-scale yellow fish (*Barbus marequensis*; Vlok & Engelbrecht, 2000).

1.4 Motivation, objectives and hypothesis

After several fruitless attempts to identify the specific cause of the crocodile mortalities, it was decided to further investigate the possible role chemical pollution may have on the reproduction of the crocodile population in the gorge, as it could have an undesirable impact on an already decreasing population. It was decided to analyse eggs of the crocodiles in the Olifants and Letaba rivers, and compare the concentrations found with concentrations in eggs from a reference crocodile farm, and eggs from a reference dam with its own catchment within the KNP.

The hypothesis is that levels of potentially toxic elements would be found in both the shells and contents of the eggs from the Olifants River and the Letaba River, and that these levels will be dissimilar to those found in the eggs from the crocodile farm and the unrelated reference area inside the Kruger National Park. The levels of organic compounds in the egg contents from the Olifants and Letaba rivers is expected to be higher than that found in the eggs from the reference crocodile farm and from the natural reference site inside the KNP.

The objectives were:

- To determine if there is a difference between the concentrations of potentially toxic metals and organic pollutants (such as organochlorine compounds and brominated flame retardants) present in the contents and shells of the eggs of Nile crocodiles from the Olifants and Letaba rivers, and compare it to eggs from a reference crocodile farm and a non-related reference area inside the KNP.
- To deduce whether the presence of the compounds and elements found may affect reproduction and thus ultimately the population of Nile crocodiles.
- To investigate if eggshell thinning occurred, and if so, the possible causes.
- To try to identify possible sources of the compounds and elements found.
- To compare concentrations of organic and inorganic elements found in the eggs from this study to those found in crocodile and bird eggs from previous studies.

2. Literature review

2.1. Metals

2.1.1. Sources

Many metals are important in our diet (as trace elements) and are also found in everyday-life in many products (LEF, 2010). However, some metals have adverse effects on humans and wildlife – most of these are known as heavy metals. “Heavy” metals are generally considered as chemical elements with a specific gravity at least five times the specific gravity of water (LEF, 2010). Certain metals cause physiological, psychological, neurological, and developmental abnormalities, as well as cancer (Nordberg *et al.,* 2007; Lavicolie *et al.,* 2009). Through experimentation it was shown that some metals can also cause disruption of the endocrine system. These metals act as hormones, or block the natural hormone activities by competing for the receptors, or influence the concentrations of a hormone (Lavicoli *et al.,* 2009). Some metal EDCs are cadmium (Cd), mercury (Hg), lead (Pb), manganese (Mn), zinc (Zn), arsenic (As), (Lavicolie *et al.,* 2009) and uranium (U) (Raymond-Whish *et al.,* 2007). Metals and metalloids have many different sources (LEF, 2010)

Heavy metals can enter streams through phosphorous fertilizers (especially cadmium), sewage pumped into rivers, industrial processes, and acid mine drainage from tailings dams (Naicker *et al.,* 2003). The concentrations of the metals from sewage depend on the composition of the treatment plant waste streams (Mortvedt, 1996; Volesky & Holan, 1995). More sources and impacts of specific metals are discussed in Section 2.1.4.

2.1.2. Accumulation

Heavy metals can enter a body through food, water, air, or absorption through the skin (LEF, 2010), and it can accumulate in the tissue (Farombi *et al.*, 2007). Many heavy metals have been found in tissues from different organs in the African cat fish (*Clarias gariepinus*; Farombi *et al.*, 2007). Metals cannot be broken down by the body, and remains until excreted. The literature on soil and water concentrations of heavy metals is extensive. Heavy metals can accumulate in soil, water, plants and animals.

Metals in tissues of crocodiles (Brazaitis *et al*, 1996) and eggs (Rainwater *et al*., 2002) have been reported. In the case of Nile crocodile eggs, the most common route of exposure is through the soil in which the eggs are laid, and through maternal transfer via the egg. The latter was found to be the case in Morelet's crocodile eggs in Northern Belize (Rainwater *et al*., 2002).

2.1.3. Associated risks

Heavy metals are known to be potentially poisonous (toxic) to almost all organisms (Giller *et al*., 1998). Their toxic characteristics are partly due to the fact that they are not biodegradable and can accumulate in aquatic organisms (Farombi *et al.*, 2007). There are at least 23 heavy metals which concerns human health: antimony, arsenic, bismuth, cadmium, cerium, chromium, cobalt, copper, gallium, gold, iron, lead, manganese, mercury, nickel, platinum, silver, tellurium, thallium, tin, uranium, vanadium, and zinc (LEF, 2010). Although these elements are available in small concentrations in the general diet and environment, intake of high concentrations may result in chronic or acute toxicity. Acute toxicity symptoms include nausea, cramping, and impaired cognitive and motor skills. Chronic toxicity on the other hand could cause slowly-progressing physical, muscular, and neurological degenerative diseases that mimic Alzheimer's or Parkinson's disease. Heavy metals can also cause cancer (LEF, 2010).

It is worth noting that vitamin E protects the body from heavy metal poisoning (LEF, 2010). In the Nile crocodile from the Olifants -and Letaba rivers, depletion in vitamin E (and selenium) was detected (WRC, 2009).

2.1.4. Metals of toxicological importance

- Chromium

South Africa was the leading producer of chromium until 2007, producing 5.6 million tons per year. The trivalent Cr plays a role in the maintenance of glucose tolerance in animals and humans (Langard & Costa, 2007). Chromium in phosphates, applied in fertilizers could be a significant source in soil, water, and food (Langard and Costa 2007). The concentration of Cr in freshwater sources is usually between 1 and 10 µg/ℓ. The absorption of Cr (III) through the digestive tract in humans is very poor, although chromates are absorbed more readily. Once it has been absorbed, Cr compounds appear in the trivalent form, until the reducing capacity of the liver is compromised after which the hexavalent form is excreted (Langard & Costa, 2007). The excretion of Cr through urine can take between 0.5 - 83 days in mice and between 15 - 41 hours in humans, but the excretion of Cr is related to its valence state. The toxicity of chromium to animals is still very much unexplored, although in some experiments ulcers have been induced in the skin and muscle tissue through application to the skin (Langard & Costa, 2007).

- Mercury

Mercury has a low affinity for oxygen, and in nature it is usually found as organometallic compounds in which it is covalently bound to carbon or organic moieties. At room temperature, elemental mercury is found in its liquid state (Berlin *et al.,* 2007). Human activity contributes to the release of mercury by combustion of fossil fuels, waste disposal, and industrial activities. Mercury has a high affinity for sulphur and sulfhydryl groups. Mercury binds to sulfhydryl groups of proteins in membranes and enzymes, interfering with membrane function and structure and enzyme activity. Mercury ions also bind to the sulfhydryl groups in albumin. For toxicological purposes, mercury can be divided into two groups: organic (methylmercury, phenylmercury, and methoxyalkylmercury); and inorganic compounds (elemental mercury and divalent mercury salts).

Methylmercury (organic mercury) occurs through methylation of elemental mercury and mercuric mercury (Hg^{2+}) in aquatic environments such as sedimentary lake beds. The methylmercury is then rapidly taken up by organisms, where it is then transformed to dimethylmercury gas ($(CH_3)_2Hg$), which enters the atmosphere. The dimethylmercury in the atmosphere is then decomposed by acidic rain, after which it returns to the aquatic system as methylmercury (Berlin *et al.,* 2007). If an inorganic form of mercury enters the body, the toxicological effects can be catastrophic, however, Berlin *et al.* (2007) reports that animals have the ability to transport and excrete this metal. If elemental mercury (mercury vapour) enters a body through inhalation, it is oxidized to mercuric mercury, which will bind to sulfhydryl groups on proteins. At high levels of exposure, mercury binds to critical nucleophillic sites and causes oxidative stress, cell injury, and even death. The uptake of organic mercury takes place through inhalation, ingestion, and absorption through the skin. The most common path for excretion is through urine. Mercury affects a number of areas such as the cardiovascular system, gastrointestinal system, liver, kidneys, and the nervous system (Berlin *et al.,* 2007). Some organic compounds of mercury are used in pesticides, and depending on the compounds, the effects (if taken up by a body) include changes in the secondary structure of the DNA and RNA molecules (Berlin *et al.,* 2007).

- Molybdenum

Molybdenum can be obtained from molybdenite ores. The primary use of molybdenum is in the alloying of metals (Turnlund & Friberg, 2007). Molybdenum is an essential element, and is readily absorbed when ingested. It is also recommended as a dietary supplement in livestock (especially sheep), to prevent copper poisoning. Absorption through the gastrointestinal tract is the major route of uptake in animals and humans. The majority of the molybdenum absorbed is distributed between the kidneys, liver, and bone in animals. Excretion primarily takes place through urine, faeces, and bile. The half-life of molybdenum is dependent on the concentration, exposure time, and the tissue affected, but it can range between 3 - 30 hours in humans, and up to 4.7 days in mice (Turnlund & Friberg, 2007). Molybdenum in natural water bodies is relatively low, but concentrations of 2 - 30 mg/kg have been found in sewage (Turnlund & Friberg, 2007).

- Palladium

Palladium is one of the platinum group metals, and South Africa is the second largest producer of it. It is used in electrical equipment, dental materials, and automobile catalysts. The natural levels of palladium in soil, water, and air are low (Satoh, 2007). The absorption of palladium mostly takes place through the trachea and then distribute to a number of organs. These organs include the kidneys, liver, spleen, blood, testis, and brain (Satoh, 2007). The primary route of excretion is through faeces. Symptoms of palladium toxicity include reduced gain in body mass, increased absolute and relative kidney mass, increased life-spans, and malignant tumours (Satoh, 2007).

- Arsenic

Arsenic is widely distributed in the earth's crust and is usually found in sulphide ores (Fowler *et al.,* 2007). Certain fish species contain very high levels of arsenic as arsenobetaine (an organic form), but many food types for humans contain some level of arsenic as well. Seafood contains higher concentrations of arsenic than any other human food source (Fowler *et al.,* 2007). Water can also contain arsenic. The marine environment contains higher levels of arsenic, although some freshwater sources may contain arsenic in up to 1 mg/ℓ, depending on the arsenic content of the bedrock. Arsenic can be found in different forms (arsenate, arsenite, methylarsonic acid, dimethylarsonic acid, etc.), and is found in both groundwater and surface water (Fowler *et al.,* 2007). Arsenic can also be found in the atmosphere as airborne dust. It is also released when copper, zinc, and lead is melted, as well as when certain chemicals and glasses are produced. Arsenic can be inhaled, ingested, and absorbed through the skin, after which it can be transported by the blood and excreted, or it can bind to the haemoglobin in the blood and be transported to the rest of the body (Fowler *et al.,* 2007). Arsenic is excreted mainly through urine. The rate at which it is excreted depends on the form of the arsenic compound. The biological half-time for arsenic in humans is 40 - 60 hours, and in mice it can be up to 60 days (Fowler *et al.,* 2007). Arsenic is a highly toxic metalloid, and there are many arsenical toxicity mechanisms. The primary mechanisms are cell injury and inhibition of mitochondrial respiration.

Furthermore, inorganic arsenicals, monomethyl arsenic acid, and dimethyl arsenic acid, have been known to cause oxidative stress.

2.2. Organic pollutants

2.2.1. Formation and major sources

Persistent organic pollutants (POPs) are organic compounds with very long half-lives in soil, sediment, and biological tissues (Jones & de Voogt, 1999), and to a varying degree resist photolytic, biological and chemical degradation (Ritter *et al*., 1995). They are hydrophobic as well as lipophilic, preferentially partitioning to lipids in organisms, and can thus be bio-accumulated (Jones & de Voogt, 1999). The mobility, persistence, and toxicity of POPs are due to their high degree of halogenation (Ritter *et al*., 1995). Compounds with these characteristics make them harmful to living organisms.

The ability of POPs to be transported long distances (in the atmosphere) before deposition can be assigned to their semi-volatility (Ritter *et al*., 1995). This means that POPs can enter the atmosphere (in gas and particulate phases) from soils, vegetation, and water bodies, where they resist (to a degree) breakdown. It is this characteristic that caused POPs to be found in places where it has not been used (Jones & de Voogt, 1999).

POPs can be either natural or anthropogenic, and include first generation organochlorine insecticides such as dichlorodiphenyltrichloroethane (DDT), dieldrin, toxaphene, and chlordane, as well as industrial chemical products or by-products such as polychlorinated biphenyls (PCBs), dibenzo-p-dioxans (dioxins), and dibenzo-p-furans (furans) (Ritter *et al*., 1995). POPs can also originate as accidental by-products from combustion, be synthesised for industrial uses (PCBs, chlorinated paraffins, and PBDEs), or used as agrochemicals (DDT, lindane, and chlordane; Jones & de Voogt, 1999). Organochlorine pesticides such as DDT are still being used to control malaria in certain parts of Mpumalanga, KwaZulu-Natal and Limpopo provinces (Bouwman *et al*., 1990). The catchments of most of the rivers of the KNP flows through DDT sprayed areas.

Another group of persistent organic pollutants is the brominated flame retardants (BFRs). These compounds can be anthropogenic and used in thermo-plastics, pharmaceuticals, pesticides, and drilling fluids (Alaee *et al.,* 2003; Yu, 2005). Organobromine compounds can also be naturally produced by marine organisms such as sponges, algae, and worms (Gribble, 1999).

The Stockholm Convention for Persistent Organic Pollutants initially identified 12 POPs; aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, toxaphene, polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans. In 2009, an additional nine POPs were added to the list. A full list and description can be found on the official website for the Stockholm Convention (SC, 2011). All POPs are halogenated organic compounds. For the purpose of this study it is important to highlight some POP compounds:

- Polychlorinated biphenyls (PCBs)

PCBs are both anthropogenic (USEPA, 2010) and formed naturally during forest fires (Gullet & Touati, 2003). PCBs were used in plasticizers in paint, capacitors, hydraulic and motor oil, rubber products, pigments, and many other applications (USEPA, 2010). PCBs are listed under Annex A with specific exemptions, and under Annex C in the Stockholm Convention (SC, 2011). PCBs find their way into the environment from poorly maintained PCB-containing hazardous waste sites, leakages from PCB containing capacitors and transformers, illegal dumping, or dumping of PCB-containing consumer products at municipal dumping sites not designated for hazardous waste (USEPA, 2010). PCBs are non-flammable, chemically stable, have a high boiling point, and have electrical insulating properties (USEPA, 2010). PCBs are known to cause cancer, and have effects on the immunological system, reproductive system, nervous system, and endocrine system (USEPA, 2010).

- Dichlorodiphenyltrichloroethane (DDT)

DDT was first synthesised in 1873 and used as an insecticide from 1939. It was extensively used in World War II for that purpose (Yu, 2008). DDT is still being used in some African countries and India for malaria control (Bouwman, 2004). It is listed under

Annex B of the Stockholm Convention (SC, 2011). DDT has a low vapour pressure, low solubility in water, and a high solubility in oils. The half-life of DDT is estimated at 7 to 30 years depending on the conditions. It is very resistant to metabolic breakdown, although in animals and humans DDT is degraded to DDE or DDD. DDT is known to have adverse effects on physiological functioning such as endocrine systems (Yu, 2005). The eggshell thinning effect of DDT has also been described (WHO & IPCS, 2002).

- Hexachlorobenzene (HCB)

HCB, like DDT, PCBs, HCH, and chlordane, is an organochlorine (Yu, 2005). It is produced anthropogenically (Stenersen, 2004). It was used as a fungicide, but is still used in some commercial closed-loop chlorination processes (Alvarez *et al.,* 2000). HCB is listed under Annexes A and C of the Stockholm Convention and has an exemption for use as a closed-process intermediate, and for use as a solvent in insecticide formulations (Bouwman, 2004). HCB is highly lipophilic (Alvarez *et al.,* 2000) and is known to bio-accumulate (van Birgelen, 1998). HCB can also enter the environment through waste incineration (van Birgelen, 1998). HCB is known to be hepatotoxic, immunotoxic, genotoxic, and is also a reproductive toxin (Alvarez *et al.,* 2000).

- Hexachlorocyclohexane

HCH was synthesized in 1825 although its pesticidal properties were unknown until 1943 (Willet *et al.,* 1998). Hexachlorocyclohexane (HCH) is an organochlorine insecticide and it is used as technical HCH (a mixture of its isomers), or as lindane, which is almost pure gamma-HCH (Li *et al.,* 2002). Alpha-, beta-, and gamma-HCH are listed under Annex A of the Stockholm Convention. These POPs were only added to the Stockholm Conventions list of POPs in 2009 (SC, 2011). A total of eight isomers of HCH are known (Willet *et al.,* 1998). Of all the isomers, gamma-HCH (lindane) exhibits the strongest insecticidal activity (Li *et al.,* 2002). The physical and chemical properties of the isomers (which are largely dictated by the axial and equatorial positions of the chlorine atoms on each molecule) vary greatly (Willet *et al.,* 1998). HCH is also known for its strong bioaccumulation potential and resistance to metabolic breakdown (Li *et al.,* 2002; Willet *et al.,* 1998). HCH is known to affect the central nervous system (CNS), liver function, and renal function. Furthermore, HCH can cause adverse reproductive effects, tumours, and are known endocrine disruptors.

- Chlordane

Chlordane is an organic insecticide mainly used to control termites (Mattina, 1999). In South Africa, chlordane was used as a pesticide. In 1993 the use of chlordane in South Africa was restricted to stem-treatment (e.g. vineyards) and construction, but all uses were stopped in 2000 (Batterman, 2008). Chlordane is listed under Annex A of the Stockholm Convention, and like HCB and mirex, it has production exemptions (Bouwman, 2004). Technical chlordane consists of 45 components. It has a low solubility in water, high solubility in lipids, a relatively low vapour pressure, and is stable under UV light. In the United States, chlordane has been detected in rainwater, soils, drinking water, air, plankton, earthworms, fish, birds and their eggs, dogs, humans, and many other organisms (Eisler, 1990). Chlordane targets the nerve and muscle membranes, resulting in membrane disruption, and ultimately death. It is also known to cause liver damage (Frear, 1955).

- Mirex

Mirex was synthesised for the first time in 1946 (Waters *et al.,* 1977). In the 1960's, mirex was used in the United States as an insecticide (USEPA, 2010a) but it has never been registered as a pesticide in South Africa (Bouwman *et al.,* 2007). Mirex is listed in Annex A of the Stockholm Convention (SC, 2011). It has been used in plastics, paint, and electrical goods as a flame retardant (Bouwman *et al.,* 2007). Mirex was recently found in bird eggs from South Africa, but at low concentrations (Bouwman *et al*., 2007). It is capable of bio-accumulation and has many adverse effects on humans and animals such as endocrine disrupting effects (USEPA, 2010a).

- Brominated flame retardants (BFRs)

BFRs are used in consumer products such as plastics and electronic circuitry to prevent fires. BFRs are normally not chemically bound, but mixed in as additives into the plastics, and are thus able to leach out into the environment (de Wit, 2002). BFRs are found everywhere in the environment. The highest levels of BFRs have often been found in aquatic wildlife (Darnerud, 2003). Many different brominated flame retardants are produced, based on the degree of bromination (Hyötyläinen & Hartonen, 2002). The toxicity of these compounds can vary, but it has been reported that they may cause cancer in humans, and play a role in disruption of the endocrine system (Rahman *et al,* 2001). BFRs are also known to have neuro-developmental effects (Hyötyläinen & Hartonen, 2002). They are persistent in the environment and can bio-accumulate (Anderson *et al.,* 2006).

Alternative additive flame retardants had to be developed due to increasing international regulations on BFRs. One such flame retardant is pentabromoethylbenzene (PBEB) which is not a Stockholm Convention POP. The literature on PBEB is scarce, but it has been detected in some environmental samples (Guerra *et al*., 2010).

2.2.2. Bio-accumulation

Because of POPs' resistance to degradation (Ritter *et al*., 1995) and their low solubility in water, they tend to accumulate in organisms at high levels even at low environmental exposure (Vallack *et al*., 1998). The most common method for uptake in the terrestrial food web is often air-plant-animal (Vallack *et al*., 1998). DDT, for example, is accumulated by humans through eating contaminated sources (Bouwman *et al*., 1990) such as produce and meat. In the aquatic environment, the methods through which the compounds enter the food web vary from absorption to ingestion of contaminated particulate manner. Organisms in higher trophic levels will most commonly be exposed to POPs through dietary intake (Fisk *et* al., 2001) or bio-magnification. The former will possibly be the route of exposure for crocodiles, as bio-concentration from water via gills is not possible. As a result of its persistence, the concentrations of POPs magnify as it moves up through the trophic levels. The bio-magnification results in the top predators in a food web with concentrations multiple times greater than in the environment (Vallack *et al*., 1998).

Different species react differently to each compound; while some species may be able to metabolise a POP, another may be less efficient. This complicates the prediction of POPs accumulation and effects (Vallack *et al*., 1998).

2.2.3. Associated risks

Persistent Organic Pollutants have many toxicological effects on animals and humans. The threat POPs poses is due to the combination of persistence, mobility, and toxicity (Ritter *et al*., 1995). The effect of POPs on an organism is influenced by the age, sex, and species, as well as the level, duration, and timing of exposure (Vallack *et al*., 1998).

In wildlife, POPs can weaken the resistance of the immune system, thereby increasing the possibility of bacterial and viral infections (Vallack *et al*., 1998). In several studies it was found that POPs can cause deformities, reproductive failure, enzyme induction, increased embryo mortalities, and many more adverse developmental and reproductive effects (reviewed by Vallack *et al*., 1998). Some POPs have been identified as having endocrine disrupting effects in wildlife (Vallack *et al*., 1998). Male alligators from eggs from a lake contaminated with endocrine disrupting chemicals (EDCs) had depressed plasma testosterone levels among other developmental effects (Guillette *et al.*, 1994; Guilette *et al.,* 1996). Organochlorine residues have been found in all seven of the 23 crocodilian species that were examined (Wu *et al*., 2000a). It is possible that EDCs can lead to reproductive complications or failure in crocodilians when exposed during embryonic development or after hatching. The organochlorine residues in crocodile eggs are most likely from maternal transfer (Wu *et al.,* 2000).

2.3. The Nile crocodile (*Crocodylus niloticus*)

2.3.1. Classification

Kingdom:	Animalia (animals)
Phylum:	Chordata (chordates)
Subphylum:	Vertebrata (vertebrates)
Class:	Reptilia (reptiles)
Order:	Crocodylia (crocodilians)
Suborder:	Eusuchia (modern crocodilians)
Family:	Crocodylidae (alligators, crocodiles and relatives)
Subfamily:	Crocodylinae (crocodylines)
Genus:	*Crocodylus* (true crocodile)
Species:	*Crocodylus niloticus* (Nile crocodile)

(Sues, 1989).

2.3.2. Evolution

Crocodiles are the only living representatives of the Archosauria (ruling reptiles) that dominated animal communities during the Mesozoic era, 245 - 65 million years ago (Sues, 1989). The Archosauria includes the crocodilians, dinosaurs, pterosaurs, and thecodontians. The crocodilians emerged in the late Triassic, at the beginning of the dinosaur domination (Buffetaut, 1989). The closest living vertebrates related to current crocodilian species are birds and lepidosaurs (Sues, 1989). The family of Crocodylidae evolved into 22 (or 23) extant species. The Nile crocodile (*Crocodylus niloticus*) is one of the largest and can grow to 5 m (Magnusson & Ross, 1989).

Evidence supporting the fossil record that birds and reptiles are related has been found in the protamines (agrinine-rich proteiens involved in spermatogenesis) of the ostrich (*Struthio camelus*) and the tinamou (*Nothoprocta perdicaria*; Ausio *et al.*, 1999). Birds, being the closest extant relative to crocodilians, also share an elongated outer-ear canal, a muscular gizzard, and a complete separation of the ventricles in the heart. Similarities in yolk deposition between reptile and bird eggs have also been shown (Astheimer, 1989). The skulls of the crocodilians (archosaurs) and the lepidosaurs (scaly lizards) share a diapsid configuration: the skull is perforated behind the eye socket by two large openings called temporal fenestrae. This allows for expansion of the huge jaw muscles. In birds, the enlarged eye sockets and the expanded brain case have encroached on the cheek region in such a way that the bone between the openings have largely disappeared. Two other features can be traced through crocodilian evolution: the development of procoelous (ball-and-socket) vertebrae from amphicoelous (spindle-shaped) vertebrae, which increases the spinal flexibility and strength; and the gradual enclosure of the secondary bony palate, which allows for breathing while the mouth is still open under water (Beffetaut, 1989).

Ferguson (1985) argues that embryogenesis and differences in haemoglobin amino acid sequences place crocodilians closer to mammals. These are just a few characteristics which crocodilians have inherited over their 200 million years of evolutionary history to become successful, amphibious hunters (Sues, 1989).

2.3.3. Habitat and distribution

With few exceptions, most crocodiles prefer tropic climates, and all of them are amphibious (Alcala & Dy-liacco, 1989). In Africa *C. niloticus* occurs from Egypt and Senegal, down to South Africa, and Madagascar (Rose, 1962). Nile crocodiles have retreated from the River Nile progressively after the 1700s (Alderton, 2009). *C. niloticus* are found in most east-flowing rivers north of 29° N latitude in South Africa (Branch, 1988). Previously found in the former Transvaal and Kwazulu-Natal in South Africa, crocodiles have been forced out by hunting, resulting in the fragmentation of populations (Branch, 1988).

Figure 2.1: Range map of *C. niloticus* (with permission from Britton, 2009)

Nile crocodiles depend on aquatic environments (such as rivers, ponds, and dams) to hunt, court, mate, and regulate body temperature. Terrestrial environments are used for nesting, moving between aquatic environments, and regulation of body temperature (Alderton, 2009). The limited ability of *Crocodylus* species to excrete salt limits their distribution to mostly fresh water habitats. The Indopacific crocodile (*Crocodylus porosus*) have adapted to more saline environments (Alcala & Dy-liacco, 1989). Nile crocodiles prefer fresh water habitats (Pienaar, 1966).

2.3.4. Feeding

Nile crocodiles, like all crocodilians, are predators. The type of prey targeted by a crocodile can be correlated to its size. This was found by investigating the stomach contents of crocodiles of various ages. There is a direct relationship between the size of a Nile crocodile and its choice of prey (Pooley, 1989). Adult Nile crocodiles' diets include fish and a variety of mammals such as rats, antelopes, and even buffalo. The larger the relative size of the prey, the longer the period between meals will become. Smaller crocodiles will feed more often, but their diet mostly consists of small fish, frogs, and insects (Pooley, 1989). Cases of cannibalism have also been recorded (Alderton, 2009).

When catching terrestrial prey, crocodiles are opportunistic, ambushing their prey while they are drinking or crossing a river. They wait until prey approaches the water, relying on camouflage and their sense of sight and smell (Pooley, 1989). Crocodiles are able to run up banks at a surprising speed for a short distance in order to grab its prey (Alderton, 2009). When striking, the crocodile will try and grab any part of its prey's body and drag it into the water. The crocodile will then attempt to drown its victim. Once dead, the carcass will be torn by violent head shakes (above water) to rip off edible pieces. Crocodiles do not chew, so the pieces are swallowed whole. The death-roll is another method by which a crocodile kills its prey and tear off edible pieces (Alderton, 2009).

When catching fish, crocodiles will try and corner their prey into an area where it is unable to escape. Using the vibrations in the water caused by the fish's movement, the crocodiles know when to strike (Pooley, 1989).

Social and cooperative feeding between crocodiles has been seen on numerous occasions. This happens when large flushes of fish appear (like the annual mullet migration in Lake St. Lucia), or when a large animal has died, big enough to feed a couple of crocodiles. The crocodiles will join in a social feed, taking turns to tear of pieces of the carcass (Pooley, 1989; Alderton, 2009). Normally, this will only happen when food is in abundance.

2.3.5. Metabolism

The metabolism of crocodiles is unique and efficient (Garnett, 1989). In a study described by Pooley (1989), the stomach contents of 30% of the investigated crocodiles were found to be empty. This is not unusual as crocodiles can go for months (even years) without feeding (Garnet, 1989). Crocodiles can do this because they don't have to actively search for food, thus very little energy is exerted. Two thirds of the energy obtained from food is stored in fat in the tail and mesenteric fat around the organs (Garnett, 1989). The stomachs of crocodiles are the most acidic of all vertebrates, with a pH value of 2, allowing it to digest almost everything consumed (even the bones and hair of mammals). The digestive process is helped along by stones in the stomach (swallowed) of crocodiles, called gastroliths (Alderton, 2009). Gastroliths are also used as a function of buoyancy, shifting the centre of gravity in the crocodile's body (Alderton, 2009).

There are costs involved with such a metabolism. One of the costs is expending energy quickly, such as when a crocodile suddenly emerges from the water during an attack on possible prey (Garnett, 1989). Because energy is normally expended slowly, there is never much oxygen in the blood. In the absence of oxygen, the levels of lactic acid will increase (Garnett, 1989). When a crocodile feeds, it will switch to anaerobic metabolism to sustain its activity. It is at this point that very high concentrations of lactic acid can build up in the blood (Alderton, 2009). Crocodilians have to rest for long periods of time after any form of energy expenditure, in order to regain normal levels of lactic acid (Garnett, 1989; Alderton, 2009). In the case of large crocodile, long struggles (energy expenditure) can lead to the death of the animal from acidosis (Alderton, 2009).

2.3.6. Reproduction

2.3.6.1. Reproductive behaviour

The mating season for the Nile crocodile in the subtropics starts in June, but intensifies in August / September as the days become longer, temperatures rise, and the rainy season starts (Magnusson *et al*., 1989). Egg laying in the subtropics of Africa occurs between September and December. Further north, the nesting period is split up into two seasons (August / September or December / January) as the temperature does not vary that much (Magnusson *et al*., 1989).

The sexual maturity of crocodilians is both size and age dependent, but males are mature at younger ages as there is a definitive sexual dimorphism, and they grow faster than the females (Magnusson *et al*., 1989). Growth of the crocodile is a function of the availability of food and ambient temperature (Ferguson, 1985).

Large males claim their territory (containing several fertile females) with aggressive behaviour, which involves the head and fore-body being lifted out of the water, swelling of the neck, and chasing adversaries. Territorial defence between males may sometimes involve biting and shaking of the tail. The submissive adversary will lift its head out of the water, and remain passive or flee to nearby sandbanks (Magnusson *et al*., 1989).

Even if a male is the victor in the battle for mating grounds, he still has to court a female. Acceptance of a courting male by a female is displayed by assuming the submissive posture (raising her head slightly out of the water, and uttering low growls). Courtship is sometimes initiated by females when they submerge their head and tail, showing only their rump (Magnusson *et al*., 1989). The male will rub its jaws on the female's head and back, and submerge to partly lift her out of the water after circling the female a few times. The rubbing releases a musk, which serves as an olfactory stimulus to the female.

Male crocodiles will mate with several females in the same period (Magnusson *et al*., 1989), although monogamy occurs in some populations (Alderton, 2009). Both sexes will travel great distances to areas with suitable nesting sites (Magnusson *et al*., 1989). Females will search for fit nesting sites long before they lay their eggs, and they will fight to obtain the best site (Magnusson *et al*., 1989).

2.3.6.2. Egg formation

Crocodilians lay eggs which are fertilised in the females (Alderton, 2009). During ovulation, the ova pass from the ovary to the oviducts where they are then fertilized. In the oviduct, the albumen part is then added, as well as the leathery membrane and the calciferous outershell (Magnusson *et al*., 1989; Alderton, 2009). The oviducts open into the cloaca, through which the eggs pass when laid (Magnusson *et al*., 1989). The eggs will, by that time, contain relatively advanced embryo's which will have reached the 20-somite stage (Alderton, 2009). A very important factor for the embryo's survival is sufficient ingredients. One such ingredient is vitamin E. A developing embryo will die if the vitamin E concentration is too low, while large crocodiles may survive (Alderton, 2009).

The literature on crocodile egg formation is very limited. Because of the evolutionary link between crocodilians and birds (section 2.3.3), and because of the similarities in egg formation (Romanoff & Romanoff, 1949), it seemed plausible to describe the formation of avian eggshells instead.

The formation of the egg (including the eggshell) is, to a large extent, controlled by the endocrine system (Romanoff & Romanoff, 1949). The eggshell is added after the yolk and albumin has been deposited. The shell-less egg is passed from the isthmus to the uterus (in the oviduct), where shell formation will start. Because the endocrine system plays an important role in the formation of the eggshell, the presence of EDCs may have an effect on the quality of the shells. Organochlorines, for example, are known to cause eggshell thinning in wild bird eggs (Lincer, 1975). *p,p*'-DDE has been reported to cause eggshell thinning under experimental conditions (Lincer, 1975). Much has been written on eggshell thinning in birds. Some examples are Lundholm, 1997; Anderson & Duzan, 1978; Faber & Hickey, 1973; Ratcliffe, 1970; and Cooke, 1973.

2.3.6.3. Egg laying

Nile crocodiles lay their eggs in holes dug in the sand along the banks of rivers and lakes. The holes are dug with their hind feet and are as deep as they can reach (20 – 30 cm). Nests are usually above the flood levels and up to 50 metres away from the water. The female crocodiles fast during the incubation period which lasts up to 3 months. They become weak and expend very little energy, although they will actively guard their nests against predators such as the Nile monitor lizard (*Varanus niloticus*; Magnusson *et al*., 1989), otters, hyenas, water mongooses, baboons, and marabou storks (Pienaar, 1966). Only half of the eggs survive predation, and as little as 2% will survive to adulthood (Magnusson *et al*., 1989).

Nile crocodiles lay 16 - 80 eggs (Magnusson *et al*., 1989) with a mean clutch size of 47.6 eggs (Thorbjarnarson, 1996). The eggs have a mean mass of between 107 g (Thorbjarnarson, 1996) to 123 g (Blomberg, 1979). The mean clutch mass of Nile crocodiles is 5 098 g, the second heaviest for all crocodilian species (Thorbjarnarson, 1996). For Nile crocodiles, there is a possible correlation between clutch mass and female size (Thorbjarnarson, 1996). Furthermore, there is a positive correlation between female size and reproductive frequency (Thorbjarnarson, 1996).

Eggs are laid in different layers, and this can have an effect on the development of the embryos, and ultimately influence the timing of the eggs hatching at the same time. To compensate, the metabolic rate (and thus oxygen consumption) of the embryo decreases as it nears hatching (after increasing during the entire incubation period). This slows the growth of the embryo and enables the eggs to hatch all at the same time. After hatching, the oxygen consumption of the crocodile will rapidly increase again (Aulie & Kanui, 1995). Because temperatures differ at different depths in sand, the layers in which the eggs are laid can affect the male / female sex ratio.

2.3.6.4. Sex determination

Sex determination in crocodiles differs from the genetic determination in other vertebrates. Temperature-dependant sex determination (TSD) has been well documented in many crocodilian species, and the critical period is between the seventh and twenty-first days (Alderton, 2009). In the case of Nile crocodiles, predominantly females will hatch at temperatures between 28 - 31°C, and 33 - 34°C. A larger male / female ratio of hatchlings will be seen with temperatures between 31 - 33°C (Lang, 1989; Alderton, 2009). The temperature inside the nests is dependent on a number of environmental factors such as ambient temperature fluctuations, rainfall, and the specific location of the nest. It is possible that one nest can deliver different sexes as the eggs are not buried at the same level, and the ambient temperature can vary greatly during the three months of incubation.

2.3.6.5. Eggshell composition

Crocodile eggshells are rigid. The calcium crystals are organised in a wedge-shaped pattern and the shells have very few pores (Packard *et al*., 1982).The shells of crocodilians consist of two layers: the shell membrane which is leathery and adjacent to the albumen of the egg; and the calciferous layer which forms the hard protective part, and covers all the contents (Packard *et al*., 1982). Ferguson (1985) also describes the two shell layers (outer calcified layer and the shell membrane), but in addition he describes three layers between those two: honeycomb layer, organic layer, and mammillary layer. The eggshells of *A. mississippiensis* consists of calcium, magnesium, and phosphorus as the main elements, but it also includes traces of copper, sodium, silicon, aluminium, iron, zinc and manganese (Ferguson, 1985).

2.3.6.6. Hatching

Yelps/calls from un-hatched crocodiles signal the female to scrape open the nest. Once the eggs are uncovered, the young crocodiles will hatch. From here they are carried in their mother's mouth to the water, where they are released. The un-hatched eggs are also taken in the mouth of the female and shaken around (Magnusson *et al*., 1989) or gently bitten until they hatch (Alderton, 2009). Hatchlings have an egg-tooth (caruncle) on the tip of their noses, which they use to slice open the eggshell (Alderton, 2009). The caruncle is shed soon after hatching (Pienaar, 1966). The female will try and protect her young from predators until they are old enough (large enough) to do so themselves (Magnusson *et al*., 1989). Inside the young crocodiles' stomachs will be remains of the yolk sack which will serve as nourishment for the first part of its development (Alderton, 2009), after which it will start preying on aquatic insects, small frogs and crabs (Magnusson *et al*., 1989).

The natural incubation environment favours the hatching of female crocodiles, which is significant in a polygamous system (Alderton, 2009). Of equal importance, when considering fluctuations in environmental factors is the possibility of a mono-sex hatching occurring under natural conditions (Alderton, 2009). This will have devastating effects on the future dynamics of the population.

2.3.7. Conservation

Crocodiles have been worshipped for a very long time (Rose, 1962). One of ancient Egypt's gods had the head of a crocodile (Trompf, 1989), and an African tribe used crocodiles to judge the guilty (Rose, 1962).

The literature on when crocodile culling began is very scarce. Apart from the skin of crocodiles, other parts like the teeth were crafted into tourist products (Rose, 1962). Thousands of crocodiles were hunted in South Africa after 1913 when the government offered a price for each crocodile killed, and for each egg collected and destroyed (Fuchs *et al.*, 1989). In 1941, culling of crocodiles was organized in the former Transvaal (Rose, 1962), and many vicious techniques of mass killings were employed (Fuchs *et al.*, 1989). Crocodiles were seen as ferocious beasts, killing and eating humans and livestock (Rose, 1962). Elsewhere, by the 1950s, nearly 60 000 Nile crocodile skins were exported from East Africa annually. Thousands more were killed for the production of candles, oil, and meat in South Africa (Fuchs *et al.*, 1989).

The South African government realized that few crocodiles were left and the species was afforded protection in 1969 (Fuchs *et al.*, 1989). The subsequent crocodile farming industries have economic and conservation importance. Although at first the need for crocodile products endangered the survival of crocodilians, the crocodile farming industry may serve as a reservoir for the re-establishment of the animals in the wild if needed (Branch, 1988).

Humans are not the only threat to crocodiles even though crocodiles are top aquatic predators. The eggs of *C. niloticus* are preyed upon by many species, e.g. the monitor lizard, marabou storks, mongoose, and honey badgers (Pooley & Ross, 1989). Furthermore, the eggs have to survive sudden changes in climate, as well as floods destroying the banks they incubate in. If they hatch, the young crocodiles are preyed upon by fish eagles, goliath herons, fish owls, and adult crocodiles (Pooley & Ross, 1989). Full-grown crocodiles have been killed by lions, hippo, and elephant (Pooley & Ross, 1989).

Irrigation farming in large areas, has contributed greatly to the geographical fragmentation of Nile crocodile populations in South Africa. In 1988, the main breeding areas were identified as being the KNP, the Limpopo River, the Olifants River, and lower reaches of rivers in the Lowveld, as well as conservation areas in parts of Kwazulu-Natal (Branch, 1988). As early as 1988, pollution was recognised as having adverse effects on the populations in the upper Olifants River, but a lack of evidence resulted in no action being taken. The majority of reasons for the endangerment of the Nile crocodile include habitat destruction, competition for resources with humans, alleged incompatibility with livestock production, the construction of dams, and over-exploitation for their skins (Branch, 1988).

3. Materials and methods

3.1. Study area

The study area (Fig. 3.1) was selected where the deaths of Nile crocodiles occurred in the KNP. The dead crocodiles were found in the Olifants Gorge and upstream in the Olifants and Letaba rivers. The Nhlanganini Dam was chosen as natural reference site. The water flowing from the dam eventually joins the Letaba River, but the catchment of the dam is not connected to the Letaba and Olifants rivers and entirely within the KNP (Fig. 3.2).

The reference crocodile farm (Castle Kop Farming, Barberton) was chosen as it did not fall within the borders of the KNP. There was no evidence of any activities similar to those found in the areas of the Letaba and Olifants rivers in the vicinity of the crocodile farm. The crocodiles on the farm are fed mainly chicken from a commercial chicken farm in the Highveld.

3.2. Sampling

In cooperation with SANParks, possible nest sites were located by helicopter. These sites were marked using a Global Positioning System (GPS). Based on the coordinates, the sites were visited on foot and the area was scouted for markings associated with Nile crocodile nests. Eggs were found by prodding around likely nest areas with a rod. The eggs were dug out of the sand by hand and wrapped in pre-cleaned tin foil and labelled according to the sites. The exact location of each nest was noted using a GPS. Detailed descriptions of each nest site were recorded. This included data such as nest size (number of eggs in the nest), general area of the nest, distance from the water, height above water level, and vegetation surrounding the nest area.

One egg was collected opportunistically from the banks of the Olifants River during field work in 2008, prior to the start of this project. Sampling for this project took place during the Nile crocodile’s breeding season (October - December) in 2009. Five crocodile nesting sites were found in the KNP (Figs. 3.2-3.3). One site was on the Letaba River and three along the Olifants River. A nest at the Nhlanganini Dam was used as a natural reference site (Fig. 3.2). Ten eggs from various ages of females were selected at the commercial farm. These eggs were not taken from the same nest, but randomly picked from the breeding chambers after they were collected. The females used for breeding on the commercial farm (Castle Kop Farming) were bought as adults from other commercial crocodile farms in Barberton and Brits (South Africa). These females were raised on their respective farms and did not originate from the wild.

All eggs collected were wrapped in pre-cleaned foil and labelled. The wrapped eggs were then kept in a cooler during transportation to the North-West University, where they were stored at -20°C.

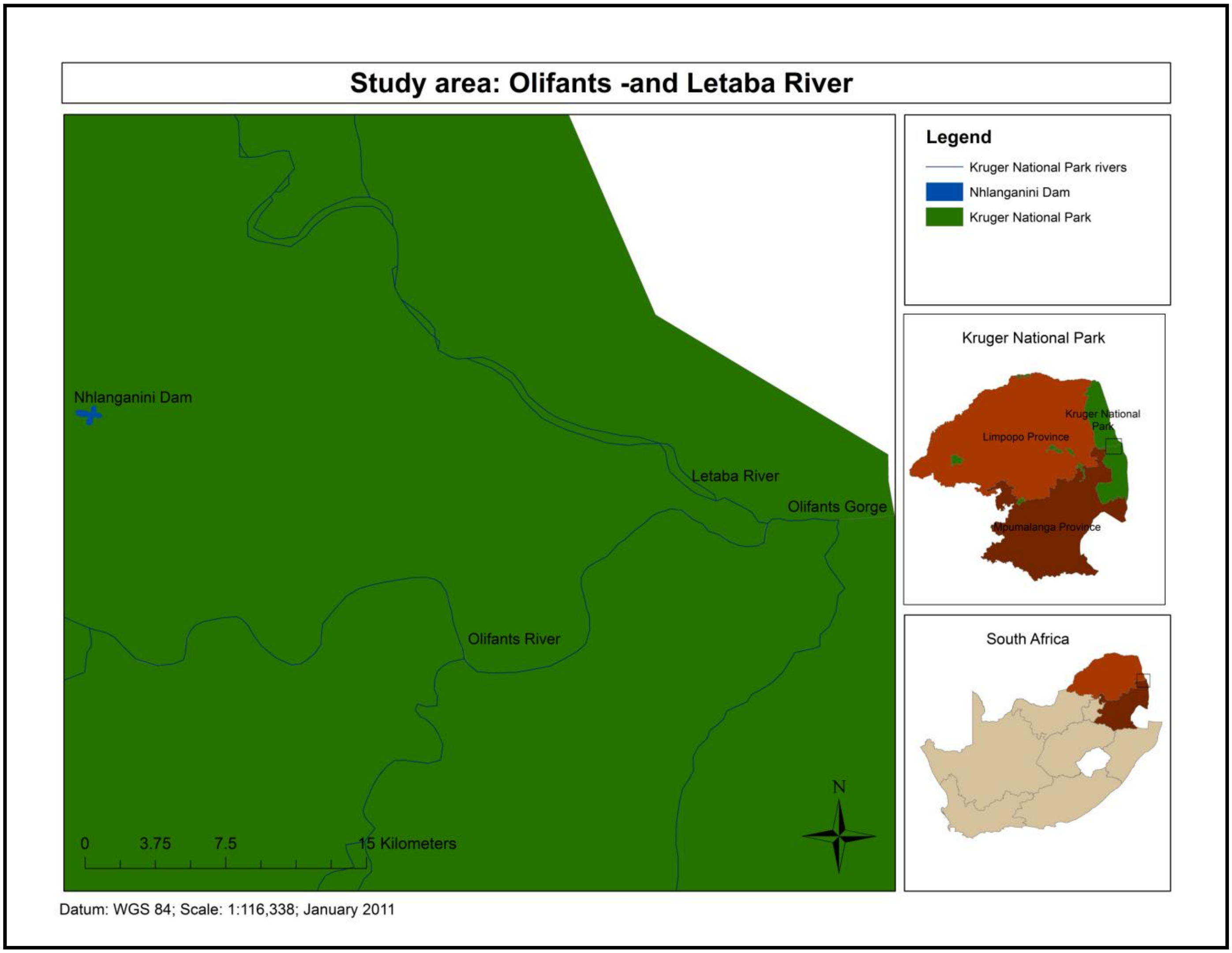

Figure 3.1: Study area in the Kruger National Park.

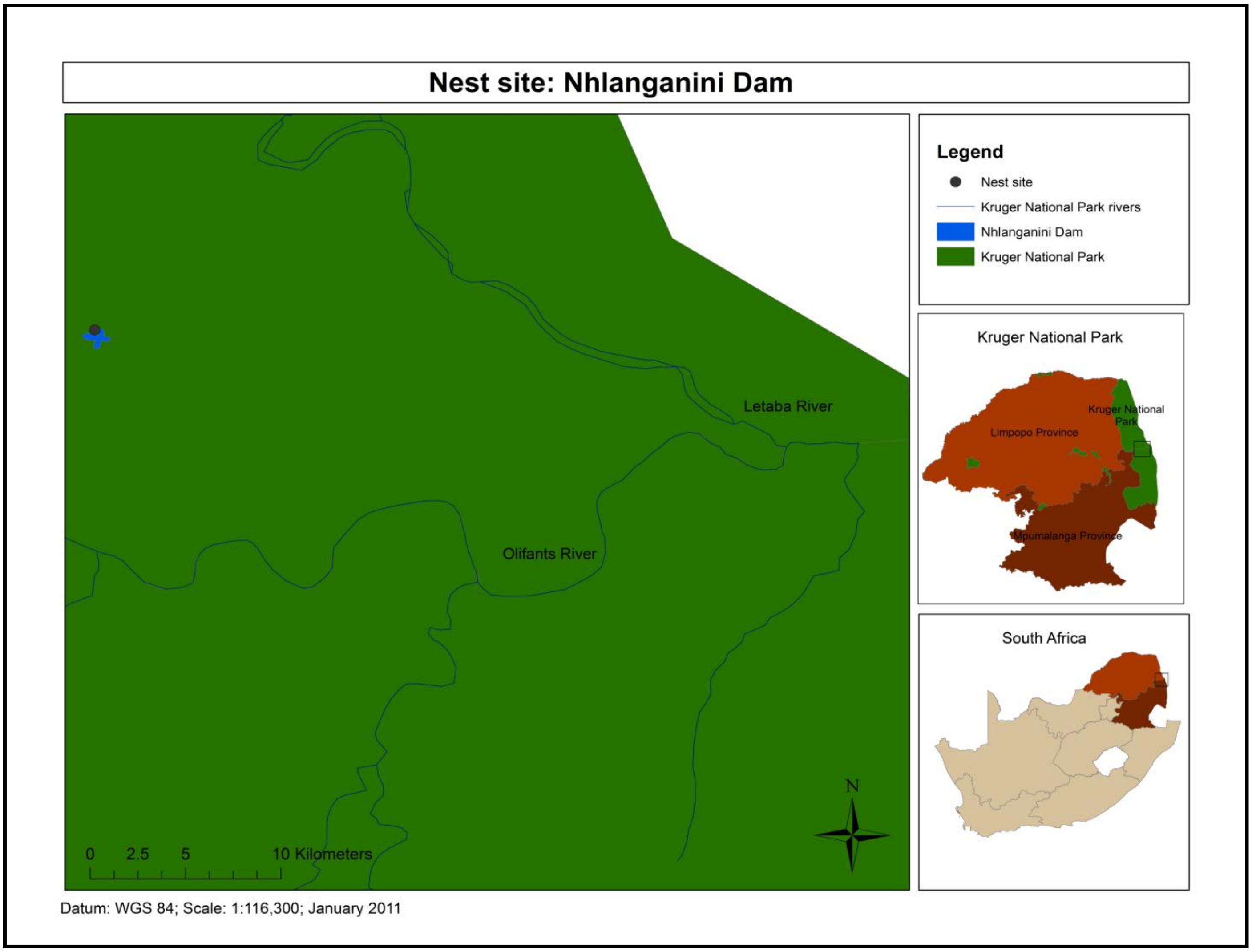

Figure 3.2: Position of the Nile crocodile nest at the natural reference site, the Nhlanganini Dam, indicated by the black dot.

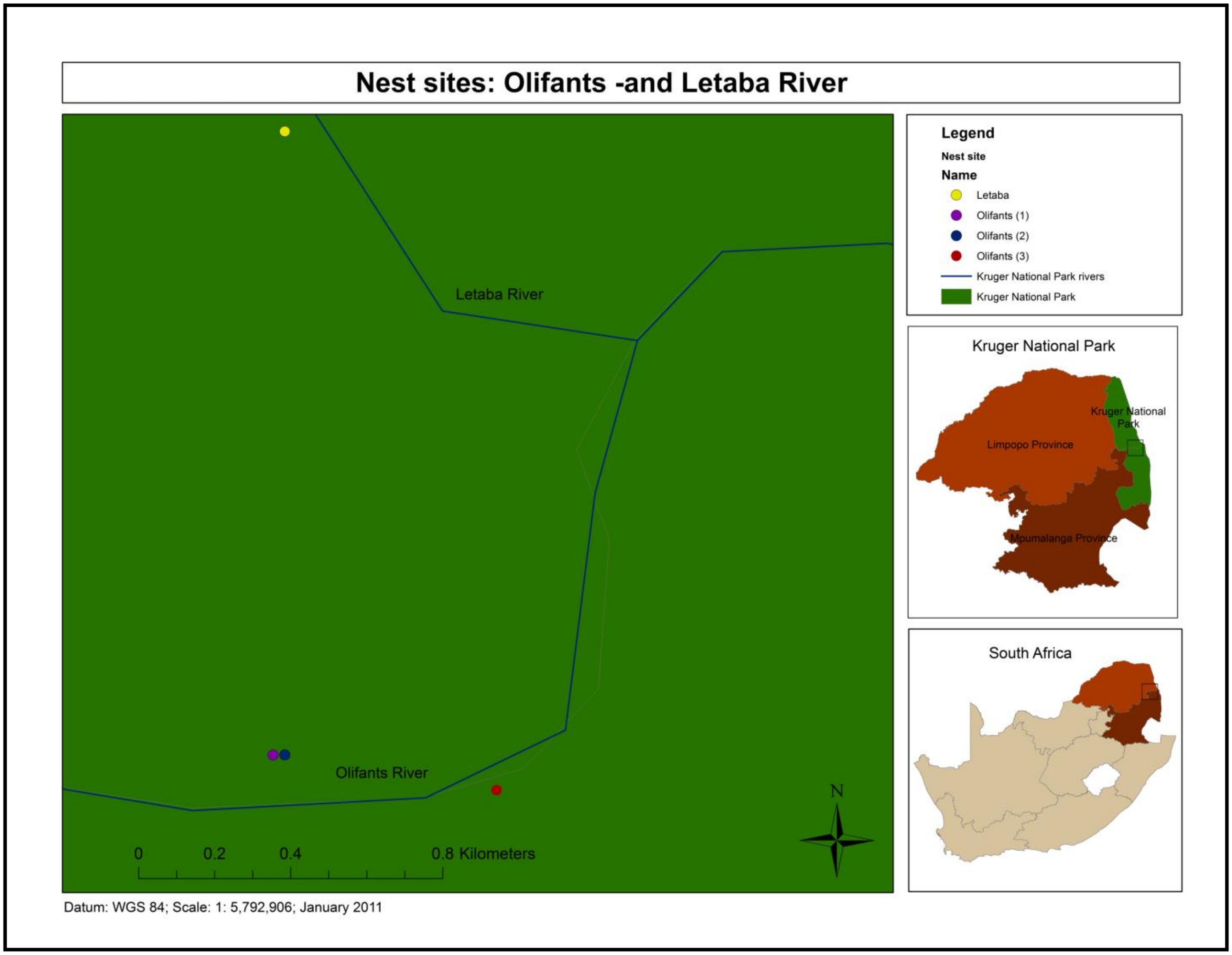

Figure 3.3: Position of the Nile crocodile nests on the Olifants and Letaba rivers, indicated by coloured dots.

3.3. Preparation and homogenization

All apparatus used in the laboratory were washed with warm soap water, rinsed three times with double distilled water, followed by separate rinses of 96% ethanol, acetone and hexane. All tools were washed before and between each sample to prevent cross-contamination. The high density polyethylene (HDPE) bottles were washed as the other apparatus, but without the separate acetone and hexane rinses.

For thawing, the frozen eggs were placed in a tin foil cup, in case the contents would leak through cracks in the shell. Each egg was weighed using a digital scale. The circumference of the eggs was measured at the widest and longest points, using a piece of string and measuring tape. The maximum length and width of the eggs were also measured, using a digital calliper.

For the homogenization process, the lights were switched off to prevent the degradation of the brominated flame retardants in the contents. The eggs were left to completely defrost in the tin foil cups. The mass of the empty, labelled HDPE bottles were recorded.

After opening the eggs, the albumin and yolk were separately placed into HDPE bottles by means of a funnel. If an egg contained an embryo, it was removed and preserved in 40% formalin, while the albumin and yolk were then homogenized using an ultrasonic liquid processor (ULP) while taking care not to form any foam. Before and between each sample, the ULP was washed as well as all other apparatus.

The homogenates were weighed. The HDPE bottles, containing the egg contents, were wrapped in tin foil (to prevent UV degradation), labelled, and stored in a freezer at -20˚C.

The eggshells were gently washed with soap water, using a soft brush to remove the membranes left on the inside. Rinsing the shells under running water removed the soap from the shells. The shells were then left for three weeks to dry in the laboratory before further analysis.

3.4. Extraction and analysis

The extraction and analysis for organic compounds was done at the Norwegian School for Veterinary Science (NVH). The analyses were conducted on the homogenized content of the eggs. The NVH has advanced analytical capacity in the field of organic contaminants (Polder *et al*., 2009), and analyzed for chlorinated organic compounds and brominated flame retardants. A complete description of the extraction procedure and analytical analysis is given in Polder *et al.* (2008) and Helgason *et al.* (2009). In short, the samples underwent liquid-liquid extraction and clean-up based on the method of Brevik (1978), modified as described by Polder *et al*. (2008) and Helgason *et al.* (2009). Due to costs, only five pooled samples were analysed for halogenated compounds (see also Table 4.1).

- Pool 1 = eggs from the crocodile farm;
- Pool 2 = eggs from the Letaba River;
- Pool 3 = eggs from the Olifants River;
- Pool 4 = eggs from the Nhlanganini Dam;
- Pool 5 = egg collected during field work from the Olifants River in 2008.

The extracts were analyzed for brominated flame retardants and organochlorines through high-resolution gas-chromatography (HR-GC) and confirmed with mass-spectrometry. The limits of quantification (LOQ) for the organic pollutants were as follows:

- For all organochlorines ≤ 0.026 ng/g wm (wet mass).
- For all BDEs ≤ 0.22 ng/g wm.
- For PBEB = 0.005 ng/g wm.

Eco-Analytica at the North-West University conducted analysis on the eggs to determine concentrations of elements using the revised international EPA 3050B method with ICP-MS, using a 2 g sample in 50 ml fluid (a mixture of HNO_3, H_2O_2, HCl, and de-ionised water) for increased accuracy. These tests were conducted on both the contents and shells of the eggs. All elements were tested for in five pooled samples (similar to the pools assigned for the organic compound tests) where after specific elements were chosen according to concentrations present in the pooled samples.

Three pieces of eggshell at exactly the same positions of the inner and outer shells were taken from each egg for measurement and analysis. The shell structure became too brittle after drying to handle and measure by hand. The measurements were therefore done with a scanning electron microscope (SEM), and each fragment was measured at five different points. Fig. 4.13 (p. 75) illustrates how the measurements were made.

3.5 Statistical analysis

3.5.1. Metals and other elements

The nest sites were divided into five pools to compare the different areas regarding concentrations of metals and elements as described in Section 3.4. The single egg in Pool 5 was not considered with the Kruskal-Wallis tests. These tests included the Dunn’s multiple comparison post-test to compare all groups with each other. The Kruskal-Wallis tests were done to determine which sites differed significantly from each other in terms of elemental concentrations. This was done for the egg contents, the eggshells, and the eggshell measurements. Data from other sources (crocodile and bird eggs) were used to compare with the data from this study using one-way ANOVA with a Bonferroni post-test to compare all groups with each other. The elements with significant differences in the Kruskal-Wallis tests were used to compare with data from other literature sources. Data from the literature which did not have standard deviations were visually compared with the data from this study.

Principal component analyses (PCA) were also done on the elemental levels in eggshells and egg contents using PCORD 5. A PCA-ordination was done on the relativised data for al multivariate analysis, except for those investigating the possible effects of elements or compounds on the eggshell thickness. The data was relativised by rows (representing individual eggs). All PCAs were done using the correlation method. This method was also used to compare the eggs from the Kruger National Park with those from the reference crocodile farm. PCORD 5 was also used to conduct multi-response permutation procedure (MRPP) to investigate the difference between the pools. MRPP tests were also done to compare the eggs from the sites in the KNP to those from the crocodile farm.

3.5.2. Organic pollutants

The pools used were the same as those for the analyses for the metals and other elements. Because there were only five samples, no statistics were applied; only visual comparisons were employed. Because of the large difference in concentrations, the data for organochlorine concentrations was split up into three groups: organochlorines not used as pesticides (PCBs); organochlorines used as pesticides, excluding DDT and metabolites; and DDT and metabolites. The sum of the chlordane isomers was calculated and presented as chlordane. The concentrations of brominated flame retardants found in the different pools were visually compared.

3.5.3. Eggshell thickness

PCA was used to investigate possible eggshell thinning associated with organic compounds and elements. The mean of the outer shell thickness and the mean of the inner shell thickness of each pool were used. Compounds that were present in only one pool were excluded. The data for the PCAs were not relativised, and the correlation method was used. The ratios of the inner and outer shell measurements (dividing the mean of the outer shell thickness by the mean of the inner shell) were also used.

4. Results

Twenty eight eggs were collected from one reference site, one natural reference nest, and four nests. Table 4.1 shows the general information of the nests found.

Table 4.1: General information of the different collection sites.

	Date	Locality	Coordinates	Nest Size	Sample Size	Pool
Croc farm (Reference)	29/10/2009	Barberton	S:25°41'15.64" E: 30°57'15.45”	various	10	1
Letaba River	17/11/2009	Letaba	S:23°58’.867 E: 31°48’.539	50-60	6	2
Olifants Nest 1	01/12/2009	Olifants River	S:23°59'53.838 E:31°48'52.073	35-40	3	3
Olifants Nest 2	02/12/2009	Olifants River	S:23°59'53.838 E:31°48'53.885	40-50	3	3
Olifants nest 3	24/11/2009	Olifants River	S:23°59'56.5 E:31°49'11.8	50-60	3	3
Nhlanganini Dam	15/12/2009	Nhlanganini Dam	S:23°55'55.565 E:31°29'48.755	25-35	2	4
Single egg 2008	04/10/2008	Olifants River in Crocodile Gorge	No data	No data	1	5

During egg content preparation, embryos of different developmental stages were found in some of the eggs. Fig. 4.1 shows examples of two of the embryos.

Figure 4.1: Two of the embryos found during the egg content preparation.

4.1. Metals and other elements

4.1.1. Elemental egg contents

The results for heavy metals and other elements found in the Nile crocodile egg contents are given in Table 4.2.

Table 4.3 shows the results of the Kruskal-Wallis tests comparing elements in egg contents in four of the five pools. The Kruskal-Wallis tests for the concentrations showed the following (up and down arrows indicate which pool had the higher or lower concentrations):

- For B, there was significant difference between the farm (Pool 1 ↑) and Olifants River (Pool 2 ↓; $p < 0.001$).
- For Mg concentration in the egg contents, there was a significant difference between the Letaba River (Pool 2 ↑) and Olifants River (Pool 3 ↓; $p< 0.05$) and between the Olifants River (Pool 3 ↓) and the farm (Pool 1 ↑; $p < 0.001$).
- For Fe, there was a significant difference between the farm (Pool 1 ↑) and Olifants River (Pool 2 ↓; $p < 0.01$), and the Letaba River (Pool 2 ↑) and Olifants River (Pool 3 ↓; $p< 0.01$).

- The Cu concentrations showed a significant difference between the farm (Pool 1 ↑) and the Letaba River (Pool 2 ↓; $p < 0.05$), and between the Letaba River (Pool 2 ↓) and Olifants River (Pool 3 ↑; $p < 0.05$).
- The As concentrations in the contents showed a significant difference between the farm (Pool 1 ↑) and the Olifants River (Pool 3 ↓; $p < 0.01$), and the farm (Pool 1 ↑) and the Nhlanganini dam (Pool 4 ↓; $p < 0.05$).
- Mo concentrations showed a significant difference between the Letaba River (Pool 2 ↓) and the farm (Pool 1 ↑; $p < 0.05$).
- Ba concentrations only showed a significant difference between the Letaba River (Pool 2 ↑) and the Olifants River (Pool 3 ↓; $p < 0.01$).

Table 4.3 was used to select the elements (those with significant differences) for further comparisons in Table 4.4. Those element used in the further comparison was limited to available literature. Table 4.4 compared some metal concentrations found in crocodile eggs in this study with those found in some earlier studies. Table 4.4 also compared this study's metal concentrations found in crocodile eggs to metal concentrations found in bird eggs of some previous studies. Concentrations are given in mg/kg dm.

Table 4.2: Concentrations of metals and elements (mg/kg dm) in Nile crocodile egg contents.

<u>Pool 1</u>	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Mean	0.5	268.03	40.34	0.76	210.66	265.92	0.279	0.020	0.055	0.076	2.46	0.002	0.021	0.345	0.72	0.038
Median	0.495	258.95	40.985	0.81	209.5	261.7	0.26	0.019	0.054	0.0705	1.76	0.0017	0.0195	0.3150	0.64	0.0365
SD	0.075	67.88	4	0.24	24.36	31.88	0.076	0.004	0.007	0.017	2.379	0.001	0.005	0.089	0.32	0.006
Min	0.42	174.8	33.85	0.23	156.9	214	0.2074	0.016	0.048	0.058	1.161	0.0014	0.016	0.27	0.1683	0.031
Max	0.62	417.5	46.33	1.1	241.4	332.9	0.48	0.028	0.072	0.11	9.162	0.003	0.032	0.58	1.212	0.047
n (10)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
% > LOQ	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

<u>Pool 2</u>	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Mean	0.41	180.12	37.22	0.797	166.8	316.85	0.27	0.019	0.055	0.070	2.22	0.002	0.019	0.26	0.49	0.031
Median	0.385	154.2	38.82	0.8	165.15	284.85	0.276	0.019	0.0555	0.065	2	0.0017	0.019	0.27	0.52	0.031
SD	0.093	68.43	3	0.19	39.59	102.60	0.021	0.001	0.002	0.009	0.86	0.001	0.003	0.042	0.15	0.002
Min	0.34	123.5	32.37	0.57	120.8	240.8	0.2463	0.018	0.052	0.062	1.37	0.0015	0.015	0.21	0.28	0.028
Max	0.59	283.5	39.42	1.1	221.7	523.2	0.2919	0.02	0.057	0.084	3.88	0.0028	0.025	0.33	0.67	0.033
n (6)	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
% > LOQ	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

<u>Pool 3</u>	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Mean	0.3	200.9	40.23	0.68	194.69	308.91	0.27	0.02	0.06	0.07	2.04	0.00	0.02	0.33	0.58	0.03
Median	0.31	192.2	39.88	0.65	181.4	306	0.28	0.02	0.058	0.061	2.211	0.0018	0.018	0.34	0.65	0.028
SD	0.03	62.64	5.20	0.27	43.16	82.57	0.04	0.00	0.00	0.01	0.72	0.00	0.00	0.06	0.23	0.00
Min	0.26	111.1	32.93	0.33	123.5	221.5	0.22	0.019	0.055	0.049	0.97	0.0012	0.015	0.23	0.28	0.027
Max	0.33	280.1	47.9	1.1	257.5	492.8	0.32	0.024	0.061	0.087	2.82	0.0022	0.025	0.4	0.90	0.035
n (9)	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
% > LOQ	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

<u>Pool 4</u>	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Mean	0.32	247.65	35.66	0.62	230.75	275.7	0.28	0.02	0.0615	0.082	2.179	0.0019	0.019	0.31	0.49	0.028
SD	0.028	11.38	0.509	0.141	3.041	1.70	0.019	0.0014	0.0021	0.0028	0.16	0.0008	0.0064	0.014	0.05	0.0014
Min	0.3	239.6	35.3	0.52	228.6	274.5	0.27	0.019	0.06	0.08	2.067	0.0013	0.014	0.3	0.46	0.027
Max	0.34	255.7	36.02	0.72	232.9	276.9	0.29	0.021	0.063	0.084	2.291	0.0024	0.023	0.32	0.52	0.029
n (2)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
% > LOQ	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

<u>Pool 5</u>	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Mean	0.29	165.8	41.01	1.2	180.7	329.7	0.33	0.05	0.074	0.08	11.19	0.013	0.024	0.46	0.83	0.028
n (1)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
% > LOQ	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 4.2: Concentrations of metals and other elements (mg/kg dm) in Nile crocodile egg contents (continued).

Pool 1	Se	Mo	Rh	Pd	Ag	Cd	Sn	Ba	Pt	Au	Hg	Tl	Pb	U
Mean	0.16	0.002	0.0067	0.0051	0.0043	0.0009	0.0057	0.34	0.0019	0.0025	0.043	0.0022	0.032	0.21
Median	0.1650	0.0025		0.0048	0.0032	0.00084	0.0053	0.36	0.0015	0.0023	0.042	0.0016	0.031	0.014
SD	0.026	0.001		0.001	0.0026	0.0004	0.002	0.076	0.0012	0.0008	0.0203	0.0019	0.0097	0.37
Min	0.12	0.0015		0.0042	0.0017	0.00061	0.0043	0.2	0.00069	0.0016	0.0013	0.0013	0.021	0.0093
Max	0.2127	0.0034		0.0071	0.009	0.0014	0.011	0.42	0.0048	0.0041	0.073	0.0075	0.049	1.1
n (10)	10	10	1	10	10	3	10	10	10	10	10	10	10	10
% > LOQ	100	100	10	100	100	30	100	100	100	100	100	100	100	100
Pool 2	**Se**	**Mo**	**Rh**	**Pd**	**Ag**	**Cd**	**Sn**	**Ba**	**Pt**	**Au**	**Hg**	**Tl**	**Pb**	**U**
Mean	0.15	0.001	0.01	0.0072	0.0029		0.0048	0.4	0.0008	0.0092	0.036	0.0012	0.023	0.012
Median	0.16	0.0013	0.0098	0.0044	0.0024		0.00	0.41	0.0008	0.0015	0.03	0.0013	0.023	0.009
SD	0.029	0.000	0.005	0.0073	0.0018		0.00066	0.032	0.000	0.019	0.003	0.000	0.005	0.009
Min	0.1	0.0011	0.0063	0.0038	0.0016		0.0037	0.36	0.00052	0.0013	0.034	0.0011	0.015	0.0065
Max	0.17	0.0017	0.016	0.022	0.0065		0.0056	0.45	0.0012	0.048	0.041	0.0014	0.03	0.030
n (6)	6	6	3	6	6	0	6	6	6	6	6	6	6	6
% > LOQ	100	100	50	100	100	0	100	100	100	100	100	100	100	100
Pool 3	**Se**	**Mo**	**Rh**	**Pd**	**Ag**	**Cd**	**Sn**	**Ba**	**Pt**	**Au**	**Hg**	**Tl**	**Pb**	**U**
Mean	0.17	0.0016	0.0086	0.0039	0.0064	0.0022	0.011	0.3	0.0025	0.0071	0.031	0.0012	0.037	0.091
Median	0.17	0.0014		0.0035	0.0032		0.0057	0.3	0.001	0.0018	0.027	0.0012	0.032	0.013
SD	0.03	0.00		0.0012	0.0052		0.013	0.03	0.003	0.013	0.0088	0.0001	0.020	0.174
Min	0.13	0.0011		0.0028	0.0021		0.0043	0.26	0.00046	0.001	0.024	0.001	0.021	0.0086
Max	0.23	0.0025		0.0067	0.017		0.043	0.36	0.0095	0.042	0.052	0.0013	0.082	0.54
n (9)	9	9	1	9	9	1	9	9	9	9	9	9	9	9
% > LOQ	100	100	11	100	100	11	100	100	100	100	100	100	100	100
Pool 4	**Se**	**Mo**	**Rh**	**Pd**	**Ag**	**Cd**	**Sn**	**Ba**	**Pt**	**Au**	**Hg**	**Tl**	**Pb**	**U**
Mean	0.22	0.0027	< LOQ	0.0033	0.008	0.00077	0.0044	0.32	0.00083	0.0011	0.023	0.00092	0.03	0.015
SD	0.014	0.0004		0.0005	0.000071	0.0001	0.001	0.02	0.0001	0.0002	0	0.00006	0.00071	0.0058
Min	0.21	0.0024		0.0029	0.0079	0.0007	0.0037	0.31	0.00076	0.00093	0.023	0.00088	0.029	0.0111
Max	0.23	0.0029		0.0036	0.008	0.00084	0.0051	0.3404	0.0009	0.0012	0.023	0.00096	0.03	0.019
n (2)	2	2	0	2	2	2	2	2	2	2	2	2	2	2
% > LOQ	100	100	0	100	100	100	100	100	100	100	100	100	100	100
Pool 5	**Se**	**Mo**	**Rh**	**Pd**	**Ag**	**Cd**	**Sn**	**Ba**	**Pt**	**Au**	**Hg**	**Tl**	**Pb**	**U**
Mean	0.13	0.0019	0	0.0028	0.0079	0.00098	0.0083	0.48	0.001	0.0011	0.025	0.0011	0.06	0.68
n (1)	1	1	0	1	1	1	1	1	1	1	1	1	1	1
% > LOQ	100	100	0	100	100	100	100	100	100	100	100	100	100	100

Table 4.3: Significant differences between four of the five pools for egg contents. Green= no significant difference ($p > 0.05$); yellow ($p < 0.05$); orange ($p < 0.01$); red ($p < 0.001$).

Element	B	Na	Mg	Al	K	Ca	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Mo	Rh	Pd	Ag	Cd	Sn	Ba	Pt	Au	Hg	Tl	Pb	U
Farm vs Letaba	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟨	🟩	🟩	🟩	🟨	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Farm vs Olifants	🟥	🟩	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟧	🟩	🟩	🟩	🟩	🟧	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Farm vs Dam	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟨	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Letaba vs Olifants	🟩	🟩	🟨	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟧	🟩	🟩	🟨	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟧	🟩	🟩	🟩	🟩	🟩	🟩
Letaba vs Dam	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Olifants vs Dam	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩

Table 4.4: Mean concentrations (mg/kg dm) of metals found in crocodile egg contents from this study, compared to that of some previous studies of crocodile and bird egg contents.

Crocodilians	**Location**	**n**	**Mercury ***	**Chromium**	**Arsenic**	**Molybdenum**	**Reference**
American alligator	Florida, USA	4	0.54				Ogden *et al.,* 1974
	Florida, USA	34	0				Heinz *et al.,* 1991
American alligator	Lake Okeechobe, USA	14		0.09	0	0	Heinz et al., 1991
	Lake Griffin, USA	12		0.08	0	0	Heinz et al., 1991
	Lake Apopka, USA	6		0.09	0	0	Heinz et al., 1991
American crocodile	Florida, USA	5	0.09				Ogden *et al.,* 1974
	Florida, USA	9	0.13				Stoneburner and Kushlan 1984
Morelet's crocodile	Belize	31	0.07				Rainwater *et al.,* 2001
Nile crocodile	Zimbabwe	26	0.23				Phelps *et al., 1986*
	Crocodile farm, SA	10	0.04	0.06	0.04	0.002	This tudy
	Letaba River, KNP	6	0.037	0.05	0.031	0.001	This tudy
	Olifants River, KNP	9	0.033	0.06	0.03	0.001	This tudy
	Nhlanganini Dam, KNP	2	0.023	0.1	0.028	0.003	This tudy
	Olifants River, KNP (2008)	1	0.025	0.074	0.028	0.002	This tudy
Birds							
Black-crowned night-heron	Hong Kong, China	9	0.52	0.318	LOQ		Lam et al., 2004
Little egret	Hong Kong, China	9	0.137	0.379	0.224		Lam et al., 2004
Brandt's cormorant	South East Farrolon, California	15	1.3				Sydeman & Jarman, 1998
Pigeon Guillemot	South East Farrolon, California	15	3.5				Sydeman & Jarman, 1998

* Compared using one-way ANOVA

Only mercury in egg contents (Table 4.4) could be analysed using one-way ANOVA for some species, as this was the only element where standard deviations were available in the selected literature. The others were visually compared using bar graphs in Fig. 4.2. The concentrations of elements in the eggs from this study were significantly lower when compared with some other studies ($p < 0.05$). When compared with the levels of mercury in the eggs of birds (Black crowned night-heron and the Little egret from Hong Kong), all four pools from this study had significantly lower ($p < 0.05$) mercury concentrations than the eggs from the Black crowned night-heron, but not lower than the eggs of the Little egret ($P > 0.05$). The crocodiles from this study had the lowest level of chromium and arsenic of all those compared (Table 4.4). Although the molybdenum levels were low, the highest concentrations were in the eggs from the Letaba River, the Olifants River, Nhlanganini Dam, and the single egg from the Olifants River collected in 2008.

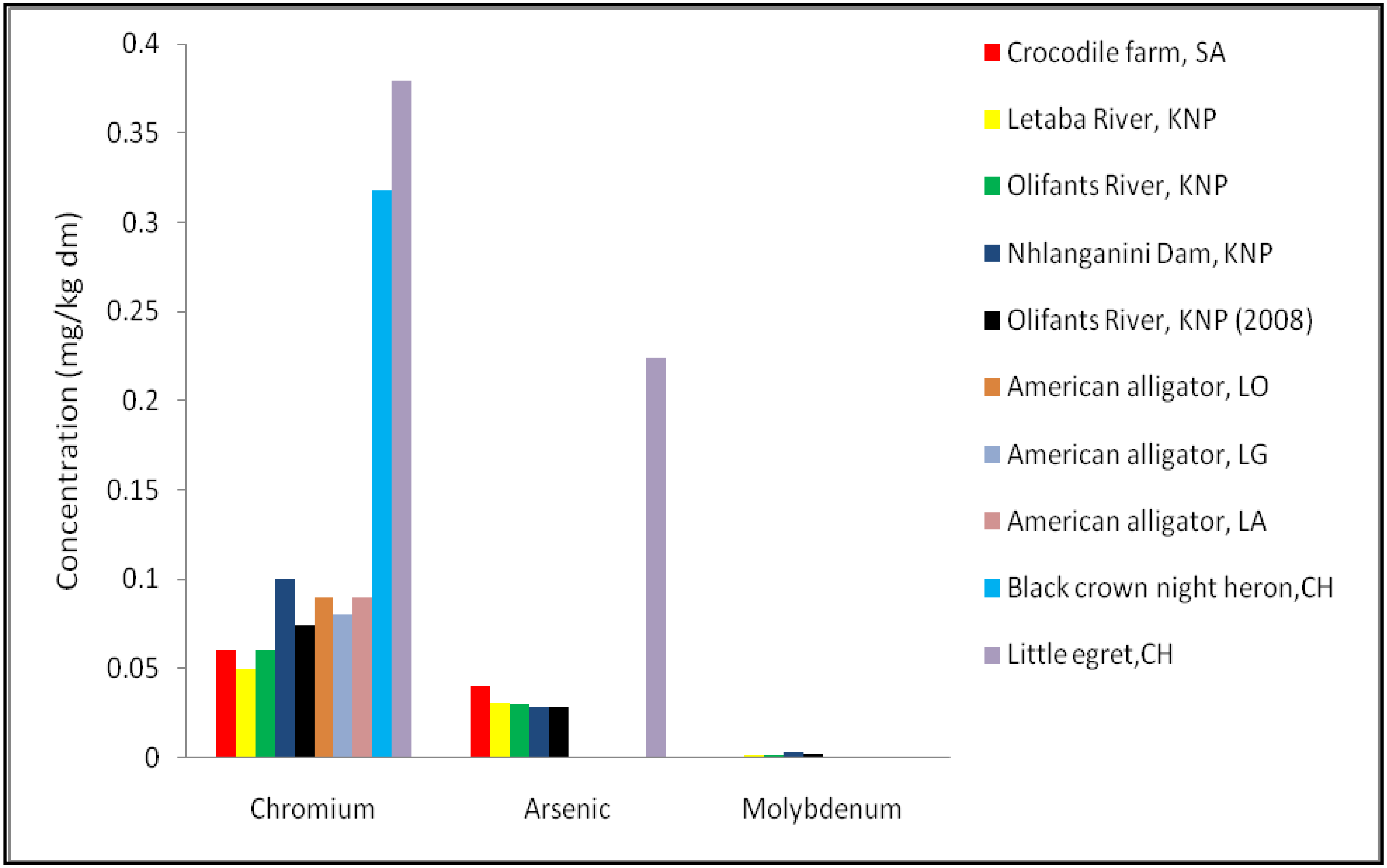

Figure 4.2: Visual comparison of concentrations (mg/kg dm) of chromium, arsenic, and molybdenum, in crocodile egg contents from this study with crocodile and bird eggs from previous studies. LO= Lake Okeechobe, LG= Lake Griffin, LA= Lake Apopka, SA= South Africa, KNP= Kruger National Park, CH= Hong Kong, China.

For the multivariate analysis, each egg was also classified according to nest number. In Fig. 4.3 the cumulative percentage description of the axes was not good (14.7%); the ordination can therefore only be interpreted in a broad sense. There are no clear clustering of eggs according to nests; the eggs towards the right had lower levels of most elements, but there were no other clear patterns. The position of most of the farmed eggs towards the right or below the origin indicated lower levels of elements such as Sn, Ag, Fe, and Co. The eggs associated with higher levels of elements were C26, C28, and C1, while C22, C16, C2 and C1 had higher levels of U. C2, C15, C9 and C1 had higher levels of elements such as Au, Ti, and Pt. Except perhaps for C24 and C25 (Fig. 4.3), there were otherwise no clear associations of individual eggs with their nests, indicating large inter-nest variation regarding elemental concentrations of the egg contents. The MRPP for the six different groups for all elements had T = -1.09, A = 0.139, and p = 0.138; therefore no significant difference in elemental concentrations between the six groups. C28 was excluded from the MRPP as it consisted of only one egg (Pool 5).

For further clarification, the eggs were classified according to those originating within the KNP, and those from outside the KNP (Fig. 4.4). The farmed eggs were mostly clustered towards the bottom and right, and the KNP eggs more towards the top, indicating some distinction. The MRPP for these two groups had the following results. T = -3.71, A = 0.18, and p = 0.01, indicating a significant difference between the two groups ($p < 0.05$). The MRPP test used, however, cannot indicate which of the elements had the most influence in this distinction.

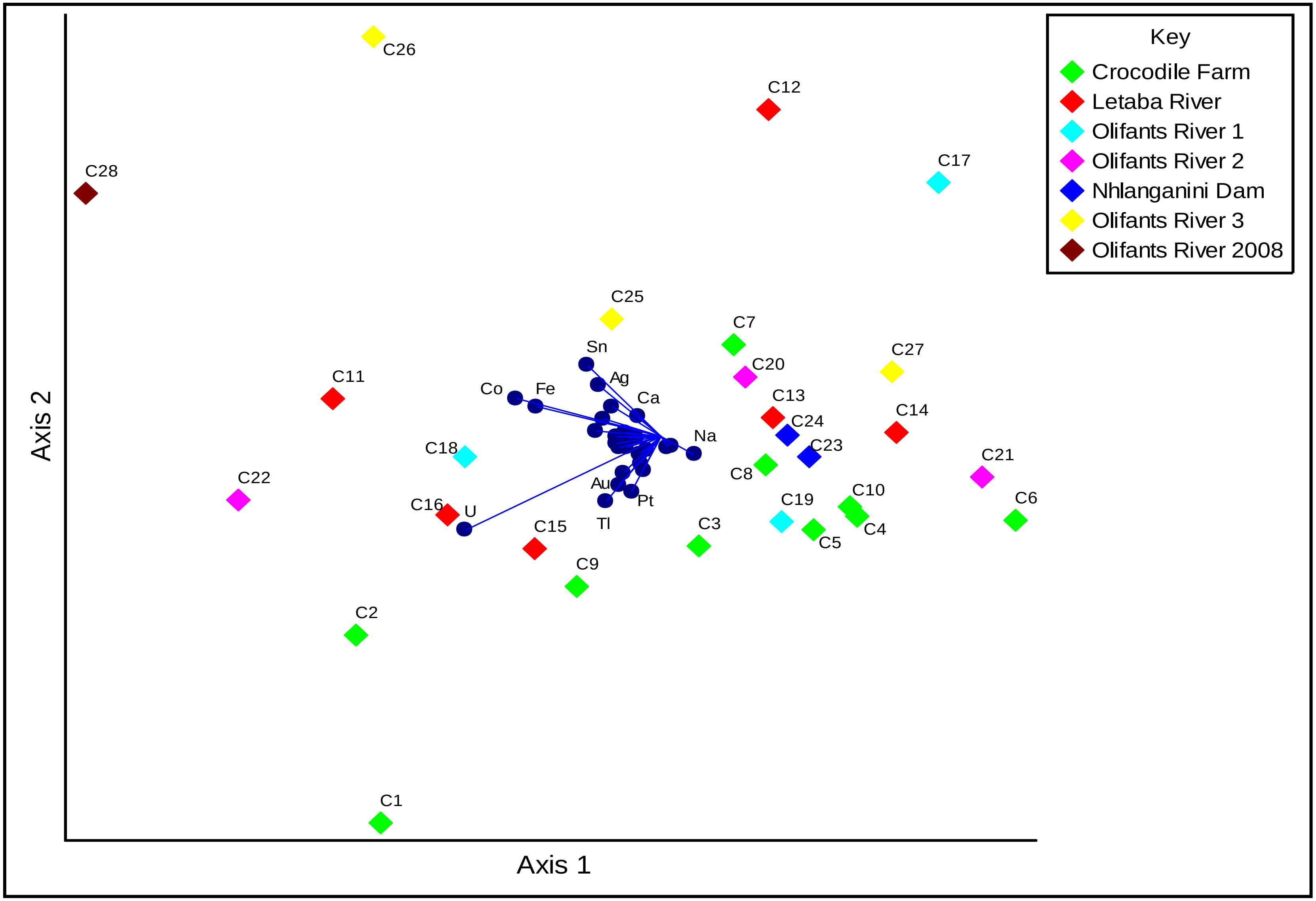

Figure 4.3: PCA plot of elements in Nile crocodile egg contents, classified according to nest site. C1-C28 indicates individual eggs. Labels of elements with short vectors are not shown. Axis 1 = 7.74%, axis 2 = 3.96%, axis 3 = 3.1%, cumulative = 14.71%. $P < 0.05$.

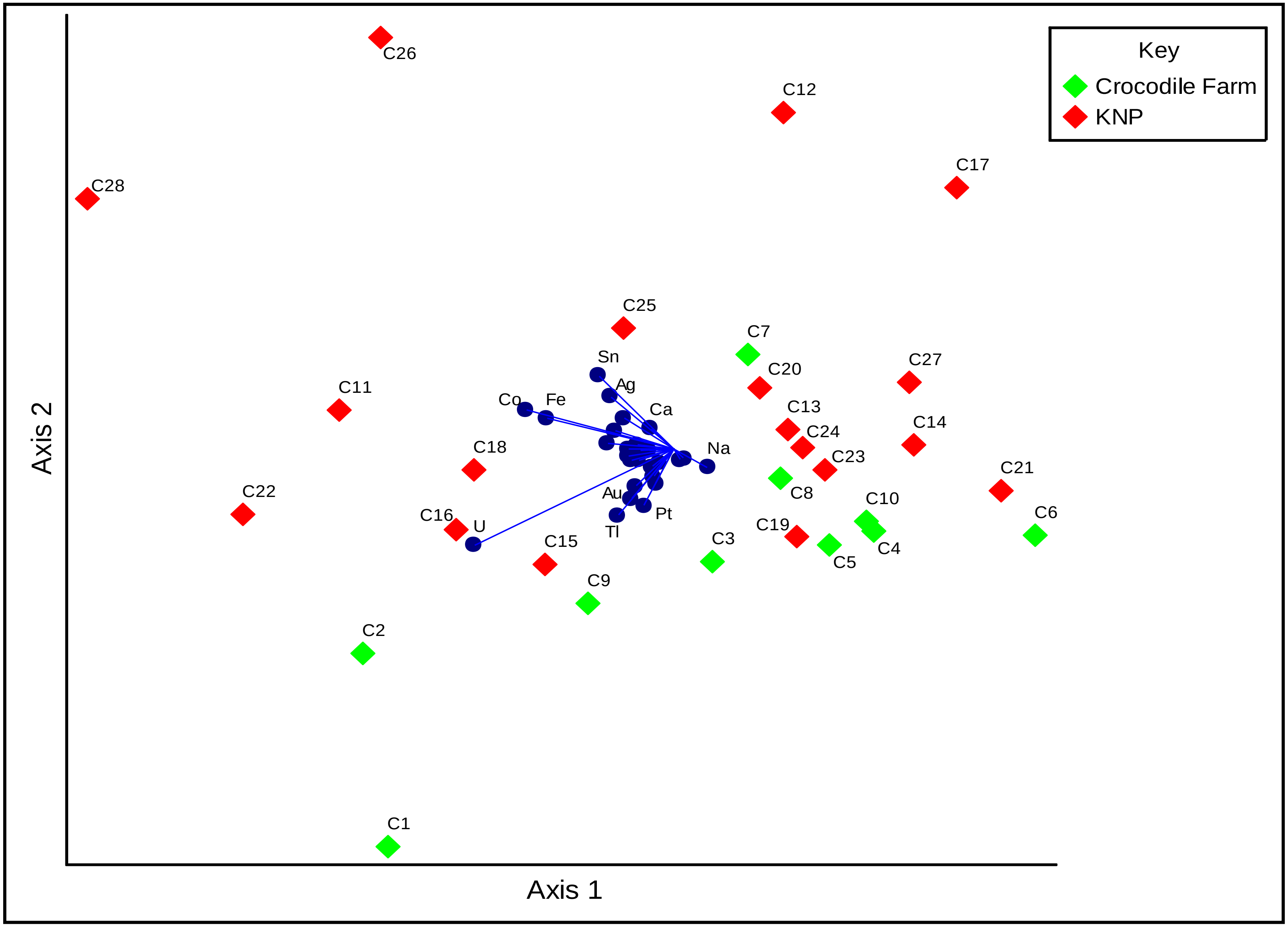

Figure 4.4: PCA plot of elements in Nile crocodile egg contents classified according to origin within and outside the KNP. C1-C28 indicates individual eggs. Labels of elements with short vectors are not shown. Axis 1 = 7.74%, axis 2 = 3.96%, axis 3 = 3.1%, cumulative = 14.71%. $P < 0.05$.

4.1.2. Eggshell elemental contents

Tables 4.5-4.6 are similar to Tables 4.2-4.3, presenting the elemental data for the eggshells. The one-way ANOVAs for the concentrations showed the following in Table 4.6 (up and down arrows indicate which pool had the higher or lower concentrations):

- B concentrations were significantly different between the farm (Pool 1 ↑) and Nhlanganini Dam (Pool 4 ↓; $p < 0.05$).
- Na concentrations showed significant difference between the farm (Pool 1 ↑) and Nhlanganini Dam (Pool 4 ↓; $p < 0.05$).
- For Cr concentrations, a significant difference was found between the Olifants (Pool 3 ↑) River and the farm (Pool 1 ↓; $p < 0.01$).
- As concentrations in the eggshells showed a significant difference between the Olifants River (Pool 3 ↑) and the farm (Pool 1 ↓; $p < 0.05$).
- Mo also showed a significant difference between the farm (Pool 1 ↑) and Olifants River (Pool 3 ↓; $p < 0.01$).
- The Rh concentrations showed significant differences between the farm (Pool 1 ↓) and the Letaba River (Pool 2 ↑; $p < 0.05$).
- Pd concentrations showed the same results as Rh for elemental concentrations in the eggshells.
- For Ba concentrations, the Letaba (Pool 2 ↑) and Olifants (Pool 3 ↓) rivers ($p < 0.01$) showed a significant difference.
- Hg and Sn showed an initial significant difference in elemental eggshell concentrations, although upon further inspection with Dunn's post-test no significant differences between the groups were found.

Table 4.5: Concentrations of metals and elements (mg/kg dm) in Nile crocodile eggshells.

Pool 1	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Mean	0.187	50.89	148.76	0.3	8.92	11508.9	0.012	0.04	0.091	0.07	68.71	0.012	0.26	0.29	0.15	0.08
Median	0.185	49.53	150.85	0.22	9.62	11605	0.0095	0.033	0.092	0.06	67.95	0.011	0.25	0.25	0.16	0.083
SD	0.009	8.64	16.15	0.28	3.86	1190.20	0.008	0.010	0.007	0.016	6.98	0.003	0.031	0.21	0.037	0.014
Min	0.18	39.75	124.6	0.06	3.3	9539	0.0039	0.027	0.076	0.054	58.77	0.0087	0.22	0.031	0.1	0.064
Max	0.21	70.64	167.8	0.99	15	13090	0.03	0.064	0.1	0.1	77.1	0.019	0.3	0.63	0.19	0.1
n (10)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
% > LOQ	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Pool 2	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Mean	0.58	50.92	113.93	0.08	13.39	10545.83	0.01	0.04	0.0987	0.068	66.63	0.01	0.27	0.32	0.13	0.1
Median	0.18	47.53	110.5	0.073	14.3	10570	0.0071	0.036	0.0995	0.067	66.77	0.0106	0.265	0.3	0.115	0.099
SD	0.99	6.28	7.93	0.071	2.76	809.62	0.0022	0.0009	0.0023	0.0088	4.99	0.00061	0.015	0.1	0.037	0.005
Min	0.15	46.39	106.2	0.01	9.79	9633	0.0056	0.035	0.094	0.058	60.12	0.0098	0.24	0.2	0.1	0.095
Max	2.6	61.1	127.2	0.18	16.2	11740	0.012	0.037	0.1	0.084	73.01	0.012	0.28	0.51	0.2	0.11
n (6)	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
% > LOQ	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Pool 3	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Mean	0.15	32.18	109.65	0.8	7.9	10191.56	0.01	0.0383	0.103	0.056	71.49	0.0117	0.3	0.18	0.1	0.107
Median	0.14	30.68	122.5	0.94	5.83	10610	0.01	0.038	0.1	0.058	75.45	0.012	0.31	0.14	0.09	0.11
SD	0.02	2.4	20.44	0.8	7.2	2376.36	0.02	0.0026	0.01	0.01	15.12	0.00	0.05	0.10	0.05	0.01
Min	0.13	30.07	67.26	0.083	2.31	5353	0.0011	0.036	0.1	0.04	35.62	0.0061	0.17	0.075	0.068	0.08
Max	0.18	36.84	126.2	2.5	26.03	12690	0.054	0.045	0.11	0.067	84.14	0.014	0.33	0.39	0.22	0.12
n (9)	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
% > LOQ	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Pool 4	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Mean	0.125	24.45	97.56	0.98	3.44	5774	0.0088	0.038	0.1	0.080	75.73	0.013	0.32	0.18	0.083	0.11
SD	0.0071	0.21	4.16	0.028	0.23	6740.14	0.0032	0	0	0.028	2.91	0.00064	0.0071	0.035	0.0007	0.0071
Min	0.12	24.3	94.61	0.96	3.28	1008	0.0065	0.038	0.1	0.06	73.67	0.013	0.31	0.15	0.082	0.1
Max	0.13	24.6	100.5	1	3.6	10540	0.011	0.038	0.1	0.099	77.79	0.014	0.32	0.2	0.083	0.11
n (2)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
% > LOQ	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Pool 5	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**
Mean	0.13	30.74	109.8	1.4	7.08	8539	0.015	0.038	0.1	0.072	67.17	0.011	0.28	0.17	0.1	0.1
n (1)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
% > LOQ	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 4.5: Concentrations of metals and elements (mg/kg dm) in Nile crocodile eggshells (continued).

Pool 1	**Se**	**Mo**	**Rh**	**Pd**	**Ag**	**Cd**	**Sn**	**Ba**	**Pt**	**Au**	**Hg**	**Tl**	**Pb**	**U**
Mean	0.07	0.002	0.03	0.0079	0.0028	0.0006	0.015	1.44	0.0010	0.0015	0.014	0.0009	0.0468	0.080
Median	0.07	0.0017	0.02	0.0079	0.0015	0	0.0073	1.323	0.00061	0.00076	0.013	0.00099	0.0375	0.013
SD	0.008	0.001	0.006	0.001	0.003	0.001	0.026	0.554	0.001	0.002	0.003	0.000	0.031	0.21
Min	0.053	0.00075	0.018	0.0058	0.00092	0	0.0031	0.97	0.00038	0.00062	0.011	0.00075	0.025	0.0098
Max	0.08	0.0028	0.037	0.01	0.011	0.0024	0.089	2.759	0.0029	0.006	0.019	0.0011	0.13	0.677
n (10)	10	10	10	10	10	4	10	10	10	10	10	10	10	10
% > LOQ	100	100	100	100	100	40	100	100	100	100	100	100	100	100
Pool 2	**Se**	**Mo**	**Rh**	**Pd**	**Ag**	**Cd**	**Sn**	**Ba**	**Pt**	**Au**	**Hg**	**Tl**	**Pb**	**U**
Mean	0.07	0.001	0.076	0.019	0.0025	0.00013	0.01	2.08	0.0009	0.001	0.015	0.00	0.02	0.02
Median	0.07	0.0012	0.075	0.019	0.0013		0.0054	2.073	0.0009	0.00089	0.015	0.00087	0.028	0.011
SD	0.011	0.0003	0.009	0.002	0.002		0.0037	0.15	0.00031	0.00056973	0.0017	0.0001	0.007	0.027
Min	0.052	0.00083	0.066	0.016	0.00084		0.0042	1.92	0.00049	0.0005	0.013	0.00079	0.015	0.007
Max	0.088	0.0016	0.09	0.021	0.0052		0.014	2.33	0.0013	0.0018	0.018	0.0011	0.032	0.078
n (6)	6	6	6	6	6	1	6	6	6	6	6	6	6	6
% > LOQ	100	100	100	100	100	17	100	100	100	100	100	100	100	100
Pool 3	**Se**	**Mo**	**Rh**	**Pd**	**Ag**	**Cd**	**Sn**	**Ba**	**Pt**	**Au**	**Hg**	**Tl**	**Pb**	**U**
Mean	0.06	0.001	0.066	0.017	0.0014	0.00008	0.0049	0.93	0.0022	0.0006	0.01	0.001	0.022	0.0084
Median	0.065	0.00077	0.07	0.017	0.00077		0.0036	0.9411	0.0014	0.00048	0.01	0.001	0.02	0.0083
SD	0.01	0.00	0.014	0.002	0.001		0.003	0.11	0.0024	0.00032	0.002	0.0003	0.0081	0.002
Min	0.054	0.00038	0.033	0.011	0.00063		0.0027	0.65	0.00038	0.00033	0.0083	0.00073	0.011	0.0059
Max	0.072	0.0026	0.081	0.019	0.0048		0.0096	1.02	0.0076	0.0014	0.015	0.0015	0.039	0.011
n (9)	9	9	9	9	9	1	9	9	9	9	9	9	9	9
% > LOQ	100	100	100	100	100	11	100	100	100	100	100	100	100	100
Pool 4	**Se**	**Mo**	**Rh**	**Pd**	**Ag**	**Cd**	**Sn**	**Ba**	**Pt**	**Au**	**Hg**	**Tl**	**Pb**	**U**
Mean	0.064	0.00099	0.071	0.018	0.00445	< LOQ	0.003	1.97	0.0013	0.0006	0.009	0.0011	0.04	0.0089
SD	0.0042	0.000021	0.001	0.0014	0.0004		0.00071	0.054	0.0013	0.00047	0.00098995	0	0.03	0.00028
Min	0.061	0.00097	0.07	0.017	0.0042		0.0025	1.93	0.00032	0.0003	0.0083	0.0011	0.018	0.0087
Max	0.067	0.001	0.071	0.019	0.0047		0.0035	2.011	0.0022	0.00097	0.0097	0.0011	0.061	0.0091
n (2)	2	2	0	2	2	0	2	2	2	2	2	2	2	2
% > LOQ	100	100	0	100	100	0	100	100	100	100	100	100	100	100
Pool 5	**Se**	**Mo**	**Rh**	**Pd**	**Ag**	**Cd**	**Sn**	**Ba**	**Pt**	**Au**	**Hg**	**Tl**	**Pb**	**U**
Mean	0.0695	0.0008	0.086	0.023	0.0005	< LOQ	0.0048	1.358	0.0012	0.0005	0.0094	0.00062	0.017	0.0063
n (1)	1	1	1	1	1	0	1	1	1	1	1	1	1	1
% > LOQ	100	100	100	100	100	0	100	100	100	100	100	100	100	100

Table 4.6: Significant elemental differences between four of the five pools for eggshells. Green = no significant difference (p > 0.05); yellow (p < 0.05); orange (p < 0.01); red (p < 0.001).

Element	**B**	**Na**	**Mg**	**Al**	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Mo**	**Rh**	**Pd**	**Ag**	**Cd**	**Sn**	**Ba**	**Pt**	**Au**	**Hg**	**Tl**	**Pb**	**U**
Farm vs Letaba	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟨	🟨	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Farm vs Olifants	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟨	🟩	🟧	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Farm vs Dam	🟨	🟨	🟩	🟩	🟩	🟩	🟩	🟩	🟧	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Letaba vs Olifants	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟧	🟩	🟩	🟩	🟩	🟩	🟩
Letaba vs Dam	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Olifants vs Dam	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩

Table 4.7 compares metal concentrations in crocodile eggshells from this study with concentrations of metals in crocodile and bird eggshells. ANOVA tests were done to compare the levels of Hg, Cr, and As. The results for Cr showed an overall p value < 0.05, a significant difference. To further clarify the results, a Bonferroni post-test was done, comparing all groups with each other. This revealed that for all pools, the concentration of Cr in the eggshells was significantly lower in the KNP eggshells than the eggshells of the Chinese alligator, except for the eggshells of the Little egret, and the crocodiles from Nhlanganini Dam. For Hg, p was < 0.0001, indicating a significant difference between the groups. The Bonferroni post-test showed that Hg concentrations in all the eggshells from other studies were significantly higher than any of the five pools from this study (p > 0.05). The As concentrations found in the different eggshells were visually compared (Fig. 4.5). The bird eggshells had the highest concentration of As of all the eggs compared. The Chinese alligator eggshells contained higher concentrations of As than any of the four pools of Nile crocodile eggshells for this study (Fig. 4.5).

Table 4.7: Mean concentrations (mg/kg dm) of elements in crocodile eggshells compared with results from other studies of crocodile and bird eggshells.

Crocodilians	**Location**	**n**	**Arsenic**	**Chromium ***	**Mercury ***	**Literature**
Chinese alligator	Chanxing	10	0.262	0.314	0.057	Xu *et al*., 2006
Nile crocodile	Crocodile farm, SA	5	0.08	0.09	0.014	This study
	Letaba River, SA	4	0.1	0.1	0.015	This study
	Olifants River, SA	5	0.11	0.1	0.011	This study
	Nhlanganini Dam, SA	2	0.11	0.1	0.009	This study
	Olifants River (2008), SA	1	0.1	0.074	0.025	This study
Birds						
Yellow-breasted chat	San Pedro, Arizona	8	1.9			Mora, 2003
	Roosevelt, Arizona	6	2.4			Mora, 2003
	Alamo, Arizina	4	2.2			Mora, 2003
	Camp Verde, Arizona	3	1.8			Mora, 2003
Willow flycatcher	San Pedro, Arizona	2	1.2			Mora, 2003
	Roosevelt, Arizona	2	1.4			Mora, 2003
	Alamo, Arizona	1	1.1			Mora, 2003
Black crowned night-heron	Hong Kong, China	9		0.085	0.056	Lam *et al.,* 2004
Little egret	Hong Kong, China	9		0.203	0.071	Lam *et al.,* 2004

* Compared using one-way ANOVAs

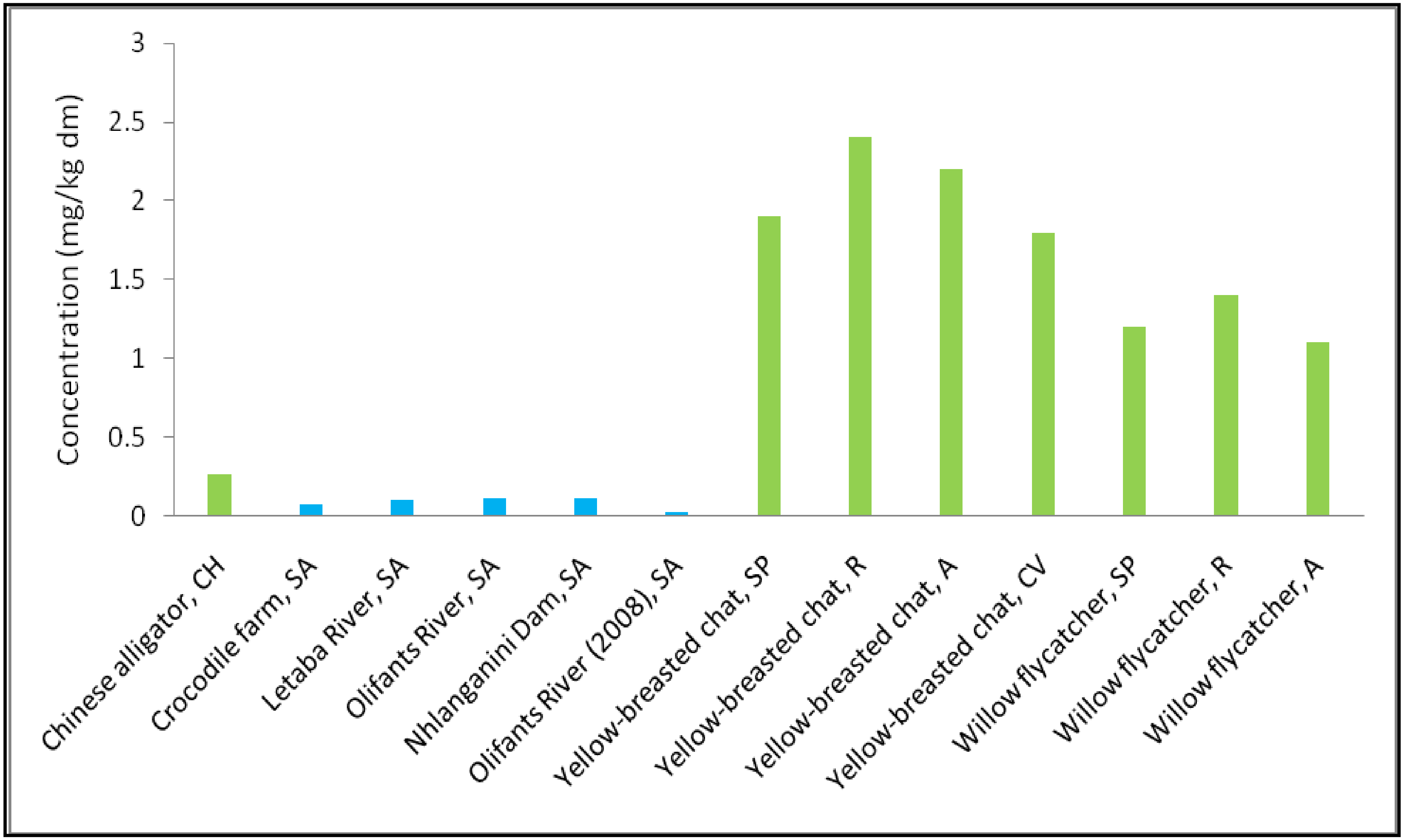

Figure 4.5: Visual comparison of concentrations (mg/kg dm) of arsenic found in crocodile eggshells for this study and previous studies of crocodilian and bird eggshells. CH = China; SA = South Africa; KNP = Kruger National Park; SP = San Pedro, Arizona, USA; R = Roosevelt, Arizona, USA; A = Alamo, Arizona, USA; and CV = Camp Verde, Arizona, USA.

The multivariate analyses for the shell elemental composition were conducted in the same manner as the elemental analyses for the egg contents (Fig. 4.6). Again, due to the low resolution obtained by the different axes, only a broad overview is possible. The eggs from each nest were clustered together in their separate nests in the ordination area. All the eggs from the crocodile farm ordinated to the top and the right of the ordination origin. The eggs from the Letaba River ordinated close to the origin. The eggs from the Olifants River ordinated to the bottom and the right of the origin, except for C26 which ordinated away from all other eggs. The two eggs from the Nhlanganini Dam were clustered together with the eggs from Olifants River nest 2. The egg collected in 2008 was clustered close to C25 and 27 from Olifants River nest 3. The eggs from the crocodile farm were associated with higher concentrations of Cd, Sn, Au, Pb, and U. C17-24 were associated with lower concentrations of the same elements. C25, 27, and 28 were associated with lower concentrations of Cd, and with higher concentrations of Pt, Rh, and Pd. C6, 8, 9, and 10 were associated with lower concentrations of Al. C11 was associated with higher concentrations of K, Zn, Co, and Ag, and C12, 13, and 16 associated with lower concentrations of the same elements (Fig. 4.6).

The MRPP for the six different groups (the egg from the Olifants River collected in 2008 could not be included in the MRPP test, as it consisted of only one egg) showed $T = -6.1$, $A = 0.57$, and $p < 0.001$, indicating a highly significant difference between the groups.

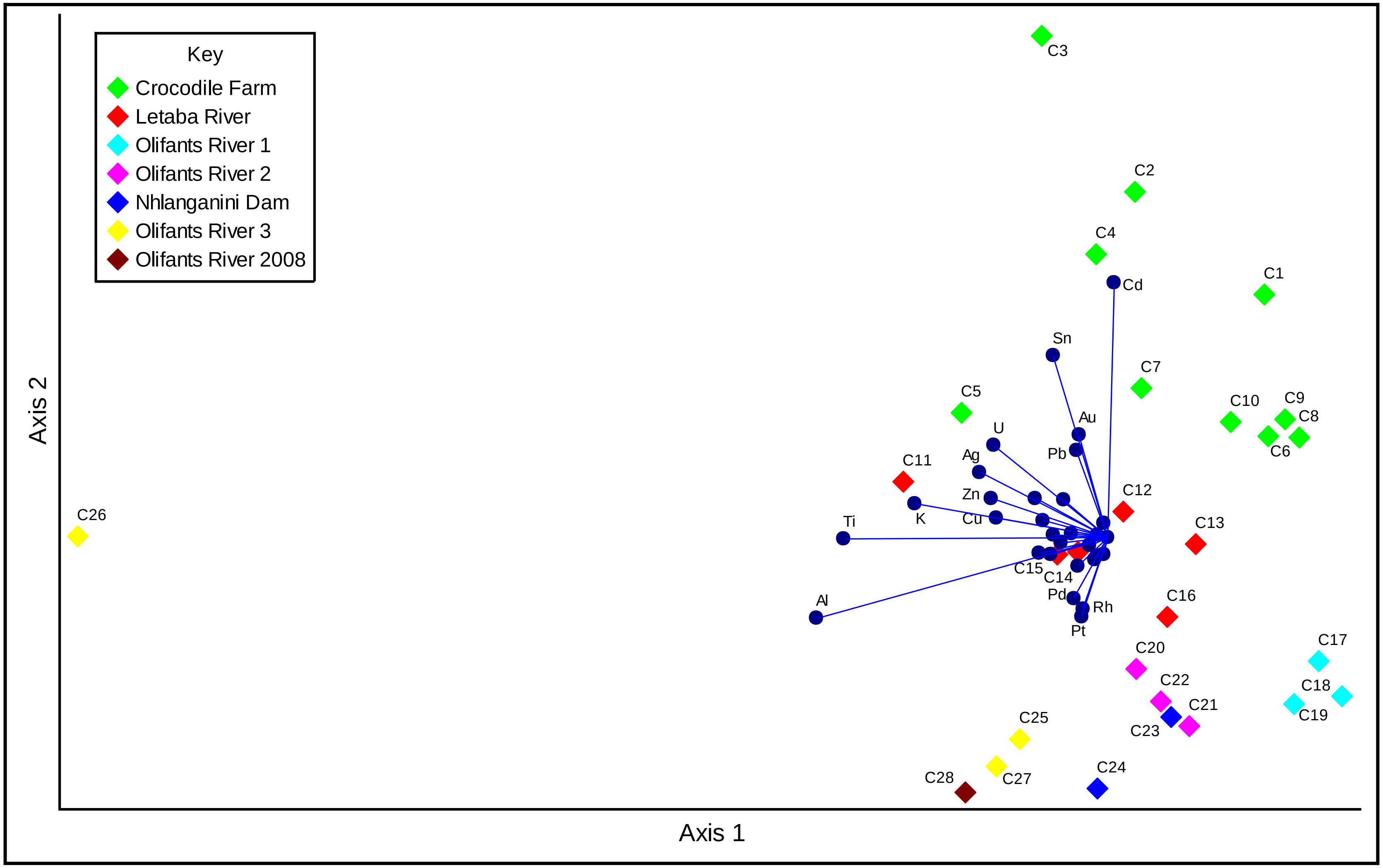

Figure 4.6: PCA plot for elements in Nile crocodile eggshells classified according to the different nest sites. C1-C28 indicates individual eggs. Labels of elements with short vectors are not shown. Axis 1 = 9.89%, axis 2 = 6.74%, axis 3 = 2.95%, cumulative = 19.58%. $P < 0.05$.

In Fig. 4.7 (classified according to origin of the eggshells inside the KNP or the farm), the separation between the KNP eggs and the eggs from the farm is clear. The eggs from the farm ordinated to the top and the right of the ordination origin, while the eggs from the KNP ordinated to the bottom and left, except for C26 which ordinated away from the rest of the eggs (Fig. 4.7). The farmed eggs were associated with higher concentrations of Cd, Sn, Au, Pb, and U. The eggs from the KNP were generally associated with lower levels of Cd, Sn, Au, Pb, and U.

The MRPP for the eggshells had the following results: $T = -12.3$, $A = 0.29$, and $p < 0.001$ indicating a significant difference between the two groups (Fig. 4.7). C28 was excluded from the MRPPs as it consisted of only one egg.

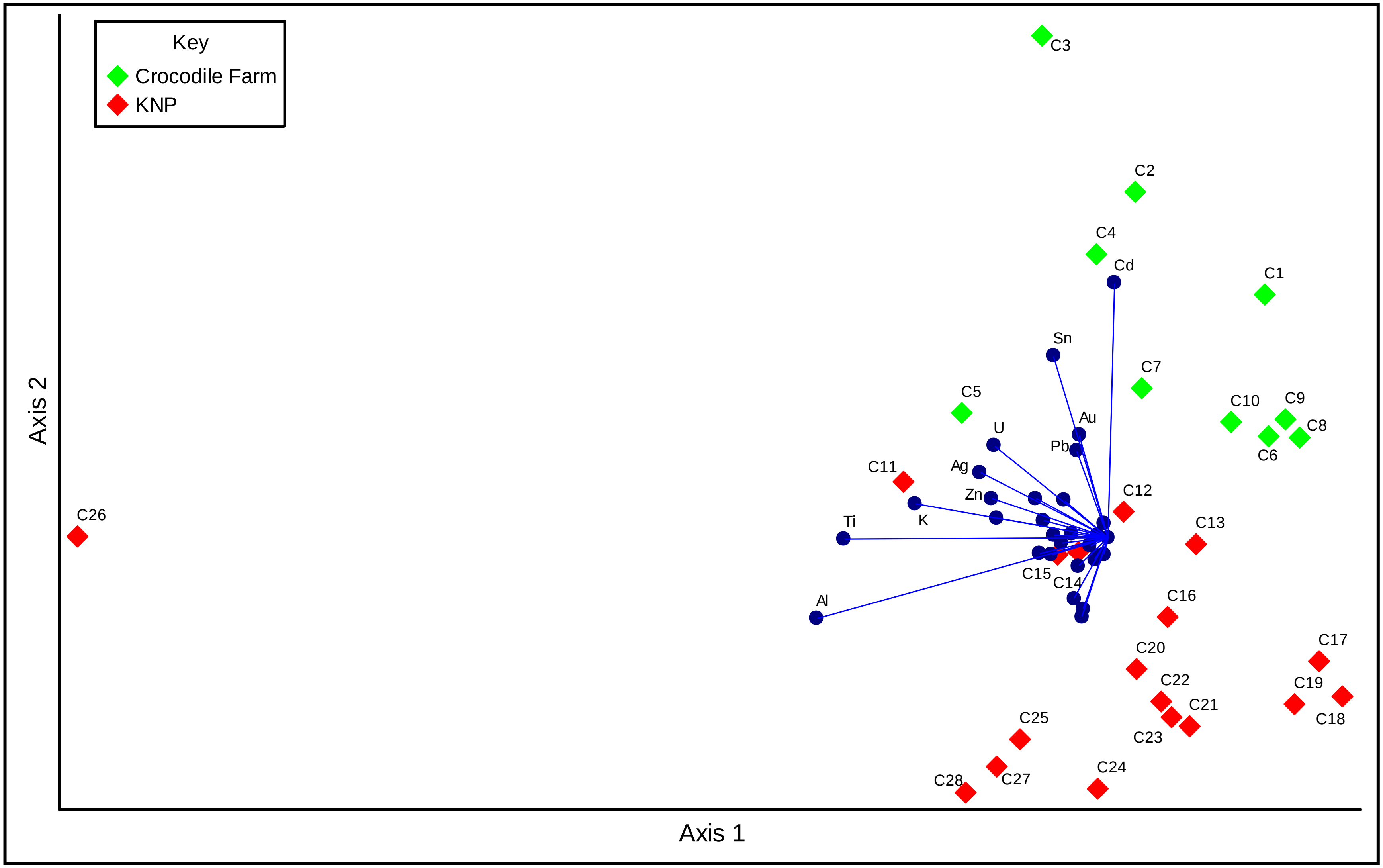

Figure 4.7: PCA plot of elements in Nile crocodile eggshells classified according to origin within and outside the KNP. C1-C28 indicates individual eggs. Labels of elements with short vectors are not shown. Axis 1 = 9.89%, axis 2 = 6.74%, axis 3 = 2.95%, cumulative = 19.58%. $P < 0.05$.

4.2. Organic pollutants in egg contents

The egg contents were analysed as five pools, each pool with an equal contribution from each egg in that pool. There were no analytical data available for individual eggs. This limited the scope for statistical analysis.

4.2.1. Organochlorines

Fig. 4.8 shows the results for all polychlorinated biphenyls found in Nile crocodile eggs for this study. Except for PCB-56, -110, -128, -151, and -199, all the PCB's analysed for showed highest concentrations in the egg from the Olifants River, collected in 2008 (Pool 5). PCB-153 was found in all pools and at the highest concentration (Fig. 4.8). ∑PCB was highest for the egg collected in 2008, and lowest for the eggs from the crocodile farm (Pool 1). ∑Organochlorines was lower in both the Olifants (Pool 3) and Letaba (Pool 2) rivers than in the eggs from the reference Nhlanganini Dam (Pool 4). Apart from DDT, β-HCH was the pesticide with the highest concentration, with the highest concentration in the eggs from the Letaba River, the crocodile farm, and the Olifants River respectively (Fig. 4.9). ∑DDT was again highest in the egg from 2008 (Fig.4.10). ∑DDT was higher for the Nhlanganini Dam than for the Letaba and Olifants rivers. The lowest concentration of DDT was in the farmed eggs. All five pools had much higher concentrations of *p,p'*- DDE than any other metabolite of DDT. The highest concentration of *p,p'*-DDE was observed in the egg from 2008, and the two eggs from Nhlanganini Dam (Fig. 4.10).

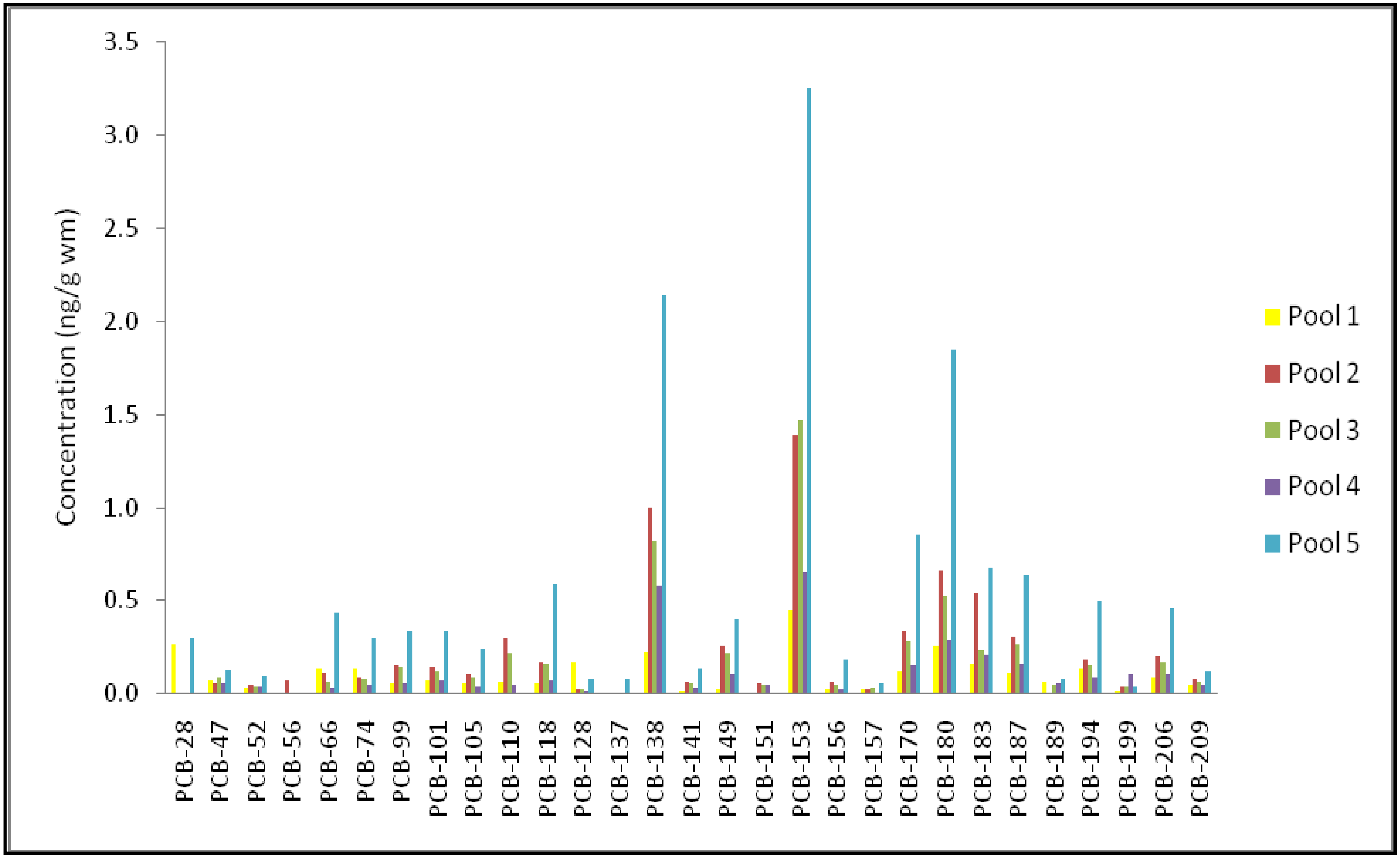

Figure 4.8: Concentrations of organochlorines (not used as pesticides) in Nile crocodile egg contents (ng/g wm). The pools are classified according to nest site. Pool 1 = Crocodile farm; Pool 2 = Letaba River; Pool 3 = Olifants River; Pool 4 = Nhlanganini Dam; Pool 5 = Olifants River 2008.

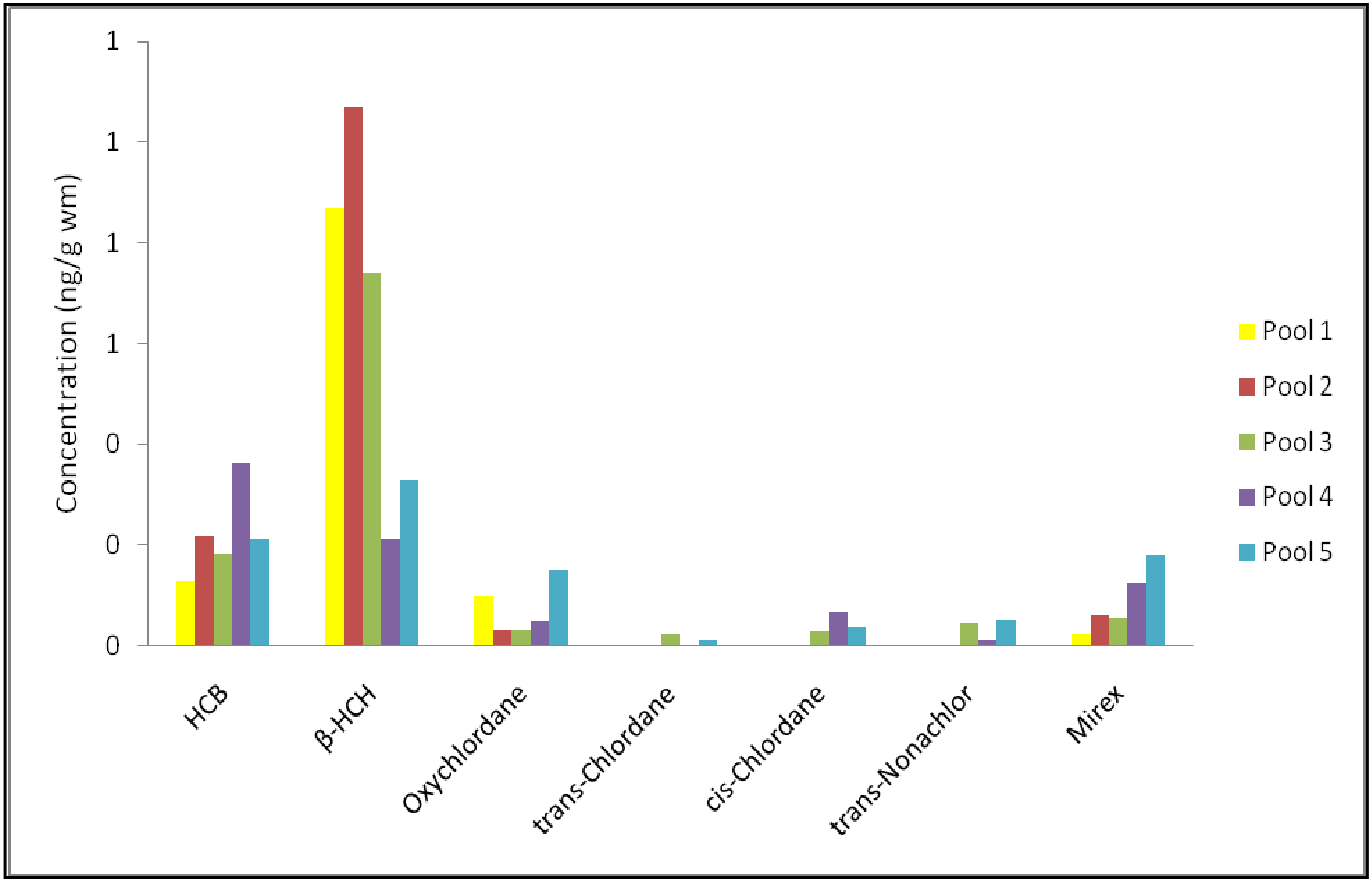

Figure 4.9: Concentrations of organochlorines (ng/g wm) used as pesticides (excluding DDT and its metabolites) found in Nile crocodile egg contents. Pool 1= Crocodile farm; Pool 2= Letaba River; Pool 3= Olifants River; Pool 4= Nhlanganini Dam; Pool 5= Olifants River 2008.

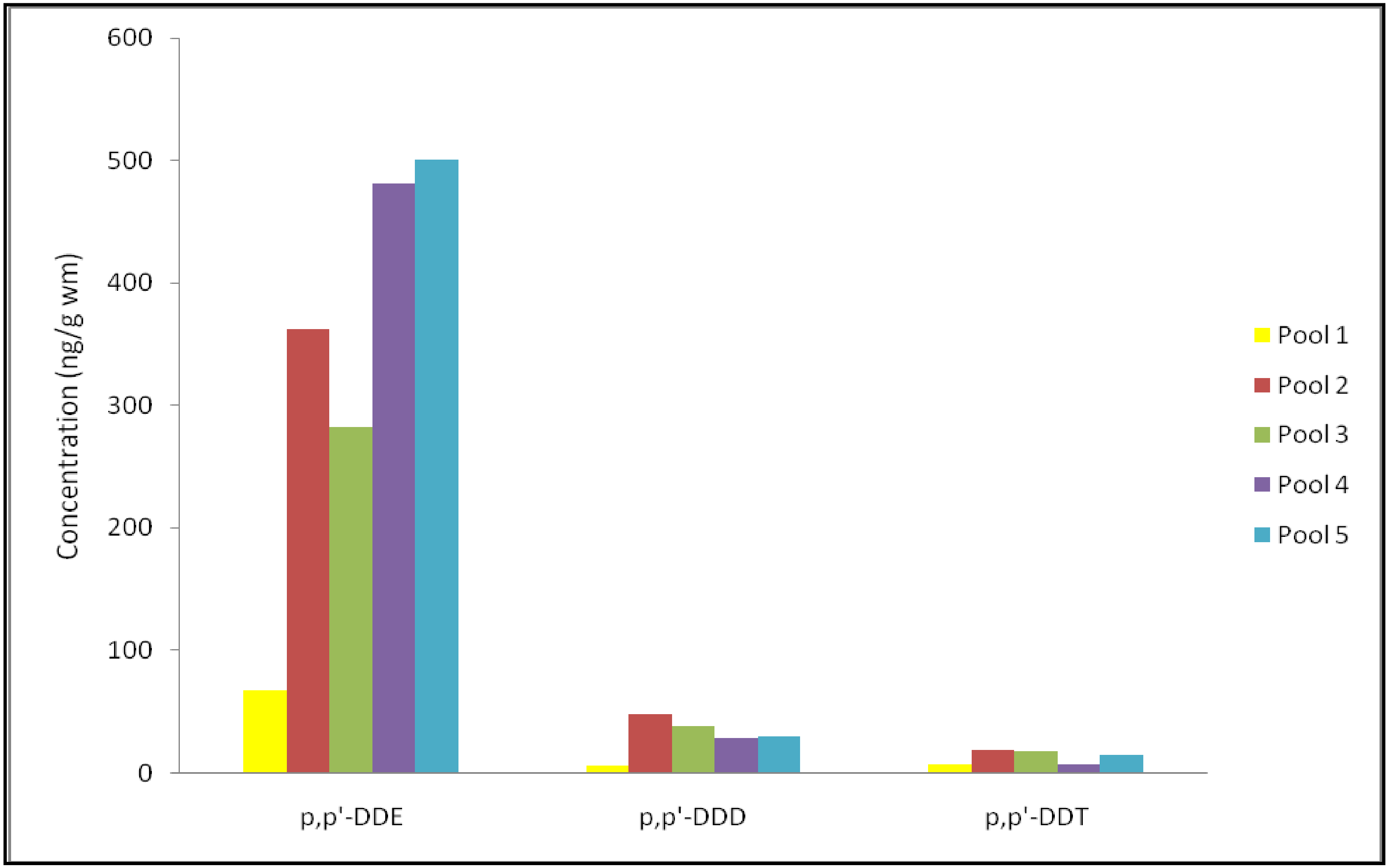

Figure 4.10: Concentrations of DDT metabolites (ng/g wm) found in Nile crocodile egg contents. Pool 1 = Crocodile farm; Pool 2 = Letaba River; Pool 3 = Olifants River; Pool 4 = Nhlanganini Dam; Pool 5 = Olifants River 2008.

4.2.2. Brominated flame retardants

For the brominated compounds, BDE- 28, -208, -207, DPTE, HBB, and BTBPE were below their respective LOQ in all pools. In Pool 1, BDE -153, -209, and PBEB were below the LOQ. BDE -99, -183, and 209 were below the LOQ in Pool 2 (Fig. 4.11). BDE -183 and –209 were below the LOQ in Pool 3. In Pool 4, only BDE -47 and -100 were found in concentrations above the LOQ. BDE -183 and PBEB were also below the LOQ in Pool 5. PBEB was found in the eggs from the Olifants (Pool 3) and Letaba (Pool 2) rivers at low concentrations. BDE -209 was found only in the egg from 2008 (Pool 5; Fig. 4.11). The egg from 2008 also had the highest concentrations of all brominated compounds tested for, except for BDE -99, -183, and PBEB (Fig. 4.11).

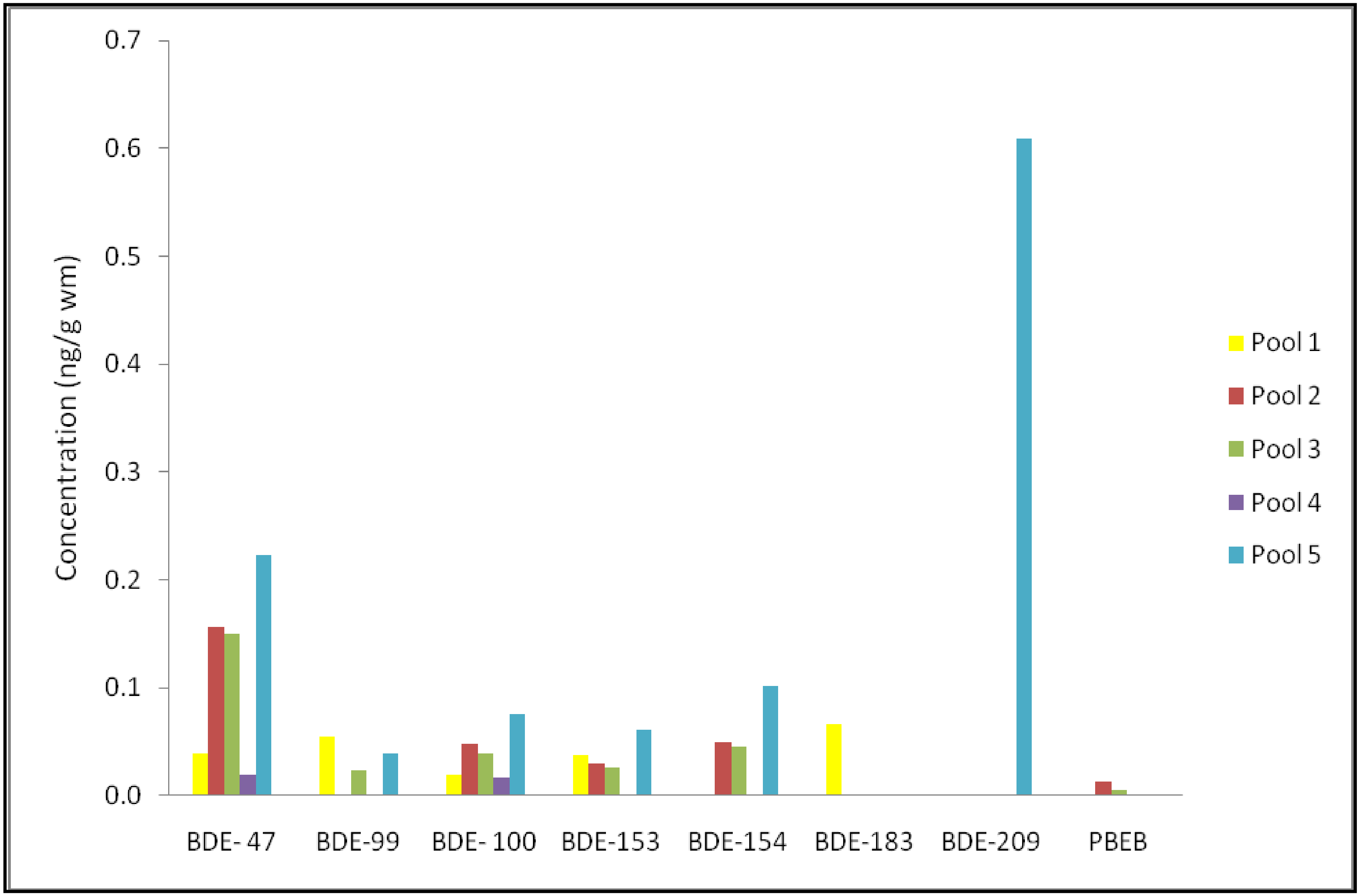

Figure 4.11: Concentrations of brominated flame retardants (ng/g wm) found in Nile crocodile egg contents. Pool 1= Crocodile farm; Pool 2= Letaba River; Pool 3= Olifants River; Pool 4= Nhlanganini Dam; Pool 5= Olifants River 2008.

4.2.3. Comparison with previous studies

The concentrations of chlorinated hydrocarbons found in the crocodile egg contents in this study were compared with the concentrations in crocodile eggs from previous studies (Table 4.8). The concentrations are presented in mg/kg wet mass.

No ANOVAs could be done to investigate the possibility of significant difference between the groups, because no standard deviations were provided, or could be calculated for the present data. The data in Table 4.8 were thus visually compared in Fig. 4.12. It is clear that the crocodile eggs from this study contained the lowest concentrations of all compounds compared with the other studies (Table 4.8 and Fig. 4.12). The highest concentration of all compounds were found in the Nile crocodile egg from a Kariba Dam crocodile farm (Zimbabwe), except for gamma-HCH which was found in its highest concentration in Nile crocodile eggs from Lake McIlwaine (Zimbabwe). Of the three groups compared (Nile crocodiles from Zimbabwe and South Africa, and Morelet's crocodiles from Belize), the eggs of the crocodiles from Zimbabwe contained the highest concentrations of all compounds. *p,p'*-DDE was the compound present in the highest concentration, and its highest concentration was in the egg from Kariba crocodile farm (Zimbabwe).

Since only values per pool were available for the organic compounds, no multivariate statistics, as for the elements, were done.

Table 4.8: Mean concentrations (mg/kg wm) of chlorinated hydrocarbons in crocodile egg contents compared with results from other studies.

Species	Location	n	α-HCH	γ-HCH	HCB	*p,p'*-DDE	*p,p'*-DDD	*p,p'*-DDT	Sum PCB	Literature
Nile crocodile	Crocodile Farm, SA	5	< LOQ	< LOQ	0.000013	0.0067	0.00063	0.00074	0.00028	This study
	Letaba River, SA	4	< LOQ	< LOQ	0.000036	0.060	0.0080	0.0031	0.00107	
	Olifants River, SA	5	< LOQ	< LOQ	0.00002	0.031	0.004	0.0020	0.00061	
	Nhlanganini Dam, SA	2	< LOQ	< LOQ	0.00018	0.241	0.0145	0.004	0.00153	
	Olifants River ('08), SA	1	< LOQ	< LOQ	0.00021	0.50	0.0296	0.0145	0.0143	
	Sengwa River clutch 17 ('81), Z	3	0.001	0.001	0.001	0.86	0.25	0.31	0.007	Phelps *et al*., 1986
	Sengwa River clutch 16 ('81), Z	2	0.001	0.001	0.001	0.49	0.15	0.17	0.008	
	Mpalangena River ('81), Z	2	0.0003	0.055	0.001	1.04	0.18	0.18	0.025	
	Chundu Island ('81), Z	2	0.006	0.024	0.001	1.29	0.4	0.331	0.022	
	Kariba Crocodile Farm ('81), Z	1	0.012	0.064	0.001	4.18	1.46	1.01	0.016	
	Lake McIlwaine ('82), Z	6	0.003	0.37	0.0003	1.76	0.37	0.34	0.32	
	Ngezi Park ('81), Z	2	0.001	0.014	0.001	0.88	0.21	0.14	0.012	
	Kyle Park ('81), Z	5	0.001	0.071	0.0004	1.43	0.34	0.31	0.054	
	Runde River 1 ('81), Z	2	0.001	0.031	0.0003	0.96	0.18	0.14	0.049	
	Runde River 2 ('81), Z	1	0.001	0.011	< LOQ	0.79	0.059	0.087	0.028	
Morelet's crocodile	GB-3 ('97), B	17				0.088	0.012	0.017		Wu *et al*., 2006
	GB-4 ('97), B	19				0.086	0.042[a]	0.012		
	GB-2 ('99), B	23				0.071	< LOQ	< LOQ		
	GB-4 ('99), B	35				0.291	0.022	0.013		
	GB-6 ('99), B	28				0.061	< LOQ	0.05[a]		
	NR-1 ('99), B	25				0.034	< LOQ	< LOQ		
	SL-1 ('99), B	28				0.194	< LOQ	0.037		

[a] Only one egg analyzed tested positive for contaminant

Z = Zimbabwe, SA = South Africa, B = Belize, GB = Gold Button Lake, NR = New River, SL = Sapote Lake.

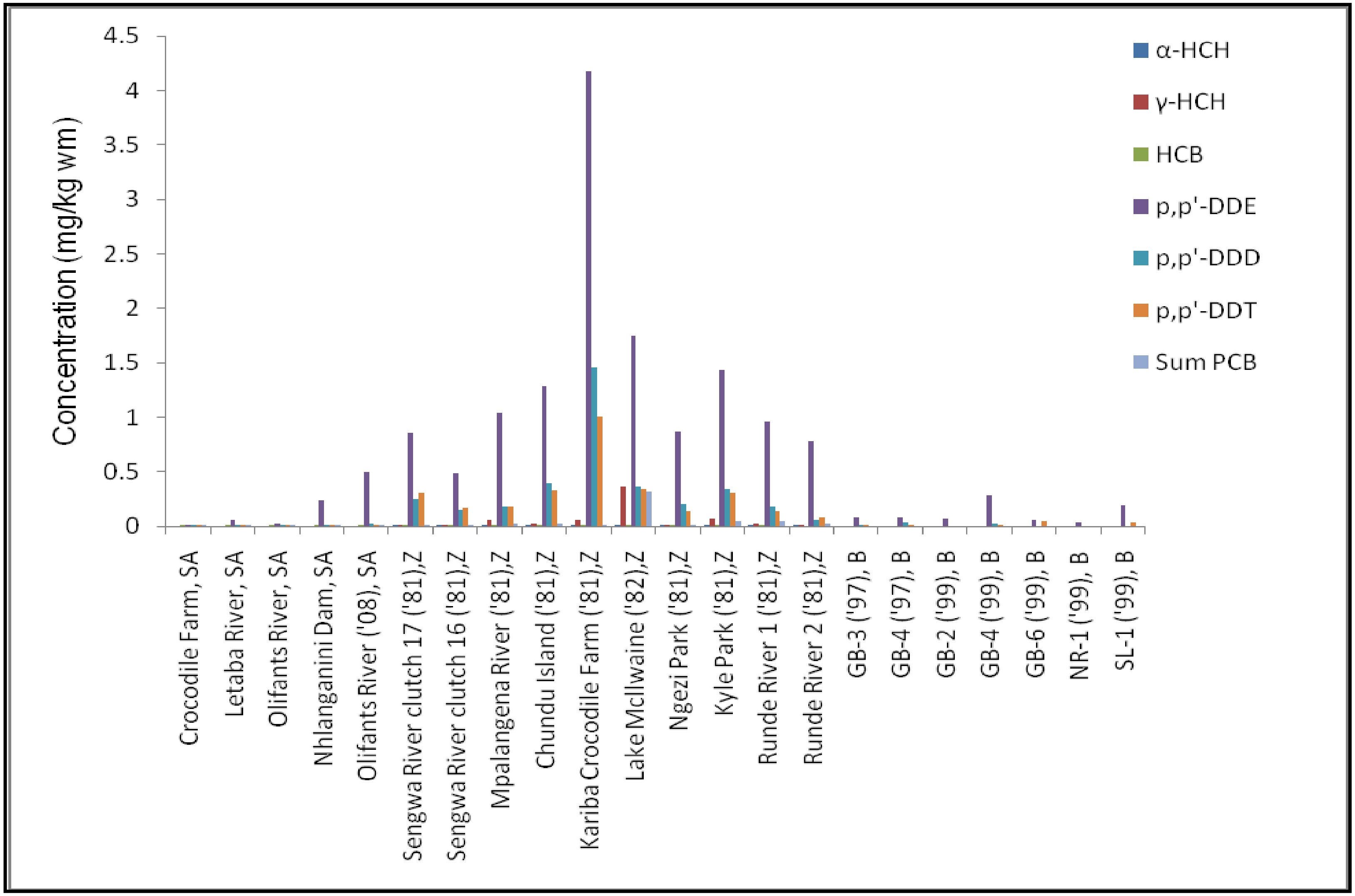

Figure 4.12: Visual comparison of concentrations (mg/kg wm) of some chlorinated hydrocarbons found in crocodile egg contents for this study and previous studies of crocodilian eggs. Z = Zimbabwe, SA = South Africa, B = Belize, GB = Gold Button Lake, NR = New River, SL = Sapote Lake.

4.3. Eggshell thickness

4.3.1. General

The results of the thickness measurements are presented in Table 4.9. Fig. 4.13 illustrates how the measurements were made using an SEM. The shells of the eggs had two clearly distinguishable layers. A porous, hard outer layer, and an inner layer which had a smooth inner surface (directed towards the contents of the egg), and a fibrous-appearing outer surface, directed towards the outer shell layer. The two layers separated easily after the shells had dried. In Fig. 4.14-15 the difference between the outer and inner shell layers can be seen. The fibrous looking material can be described as what is called the mammillary layer and the organic layer by Ferguson (1985). These fibres possibly bind the inner and outer layers.

Kruskal-Wallis tests (with Dunn's post-test) were done to compare the thickness of each layer of Nile crocodile eggshell per pool, using the data in Table 4.9. The tests conducted for the outer shell layer showed an overall $p < 0.0013$, indicating a significant difference between the pools. Using the Dunn's post-test, a significant difference was found between the eggs from the farm (Pool 1) and the Olifants River (Pool 3; $p < 0.01$). The same tests for the inner shell thickness revealed no significant differences between any pools ($p > 0.05$). For the combined outer and inner shell thickness (therefore total eggshell thickness), the tests showed $p < 0.0079$. Upon further inspection with the Dunn's post-test, the only significant difference was between the crocodile farm (Pool 1) and Olifants River (Pool 3; $p < 0.05$).

Table 4.9: Thickness of Nile crocodile eggshells (µm)

Measurement	Mean of outer shell and inner shell	Mean of outer shell	Mean of inner shell
Egg ID			
C1	315.1	413.2	217
C2	290.7	376.4	205
C3	324.0	443.4	204.6
C4	314.4	394.7	234
C5	228.4	318.8	138
C6	247.2	358.4	135.9
C7	232.2	325.4	139.1
C8	257.2	361	153.3
C9	268.9	348.6	189.2
C10	241.3	330.2	152.4
C11	261.2	365.8	156.5
C12	280.6	388.6	172.5
C13	313.5	406.4	220.7
C14	285.3	373.3	197.3
C15	284.4	370.4	198.4
C16	298.6	389.6	207.6
C17	345.7	497.4	194
C18	345.5	501.8	189.2
C19	341.1	491.9	190.3
C20	296.7	421.4	172.1
C21	321.2	427.6	214.7
C22	297.2	434.2	160.1
C23	328.7	444.0	213.3
C24	326.8	461.5	192.1
C25	304.7	427.9	181.6
C26	325.4	408.1	242.7
C27	294.0	399.2	188.8
C28	301.4	423.8	179

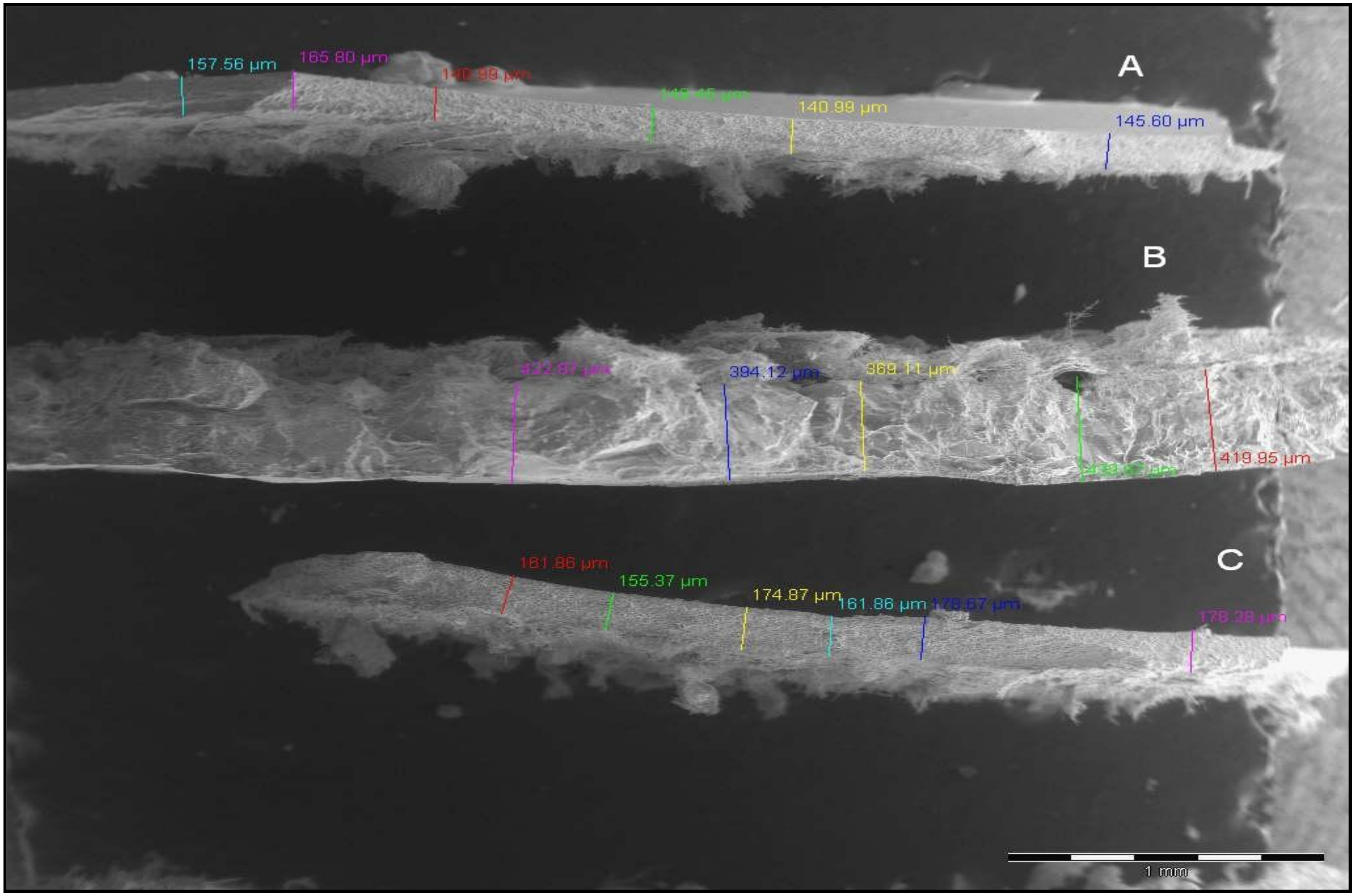

Figure 4.13: SEM micrograph showing the measurements of the thickness of crocodile eggshell fragments. The micrograph was taken of fragments of one egg to illustrate how the measurements were conducted on all crocodile eggshell fragments with three fragments per egg. A = inner layer; B = outer layer; C = inner layer. An average of five measurements was made on each fragment.

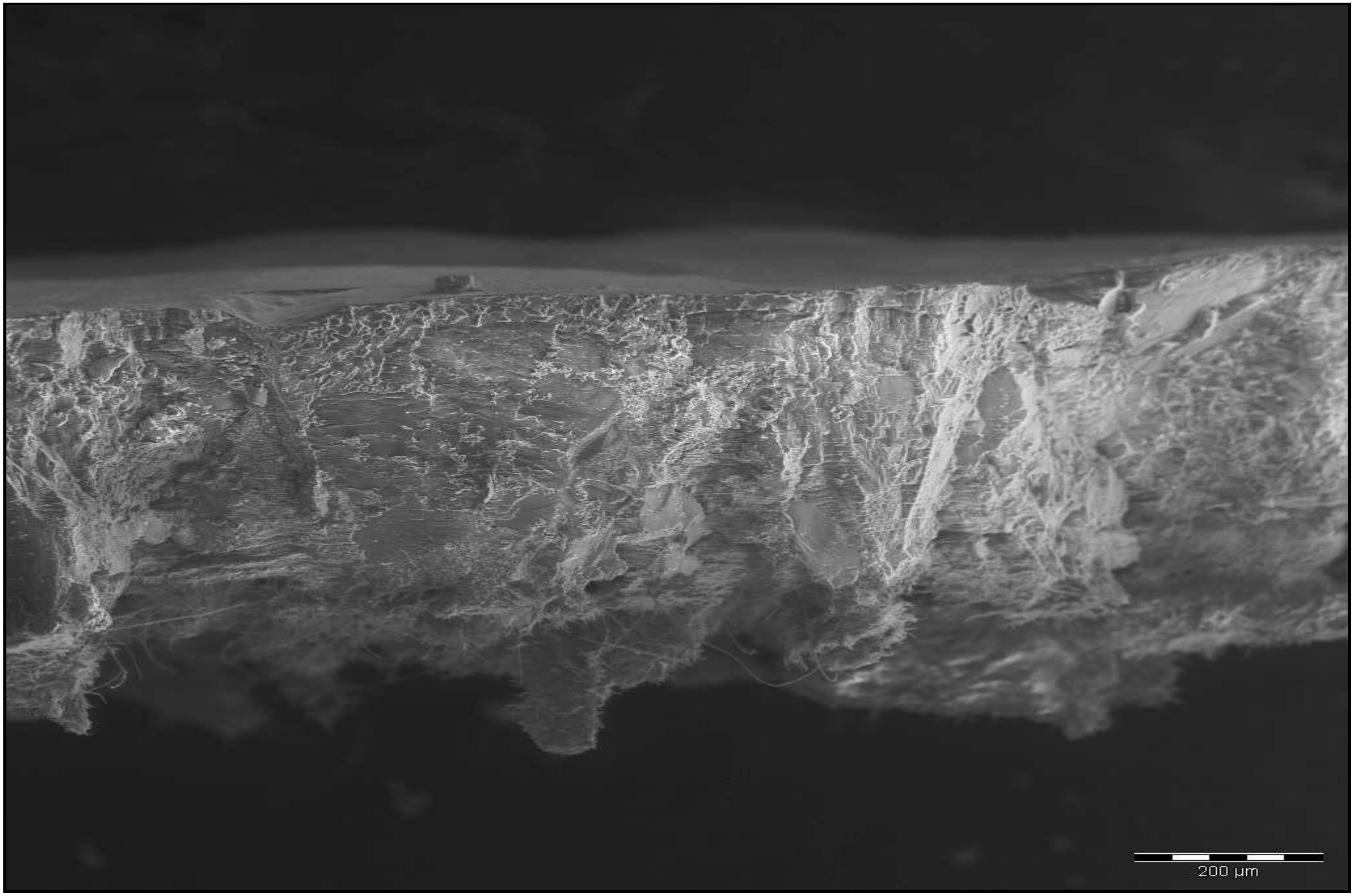

Figure 4.14: SEM micrograph of outer eggshell layer fragment of Nile crocodile. The smooth surface is directed towards the surface of the egg, away from the embryo, and the rougher surface is directed towards the inner shell layer.

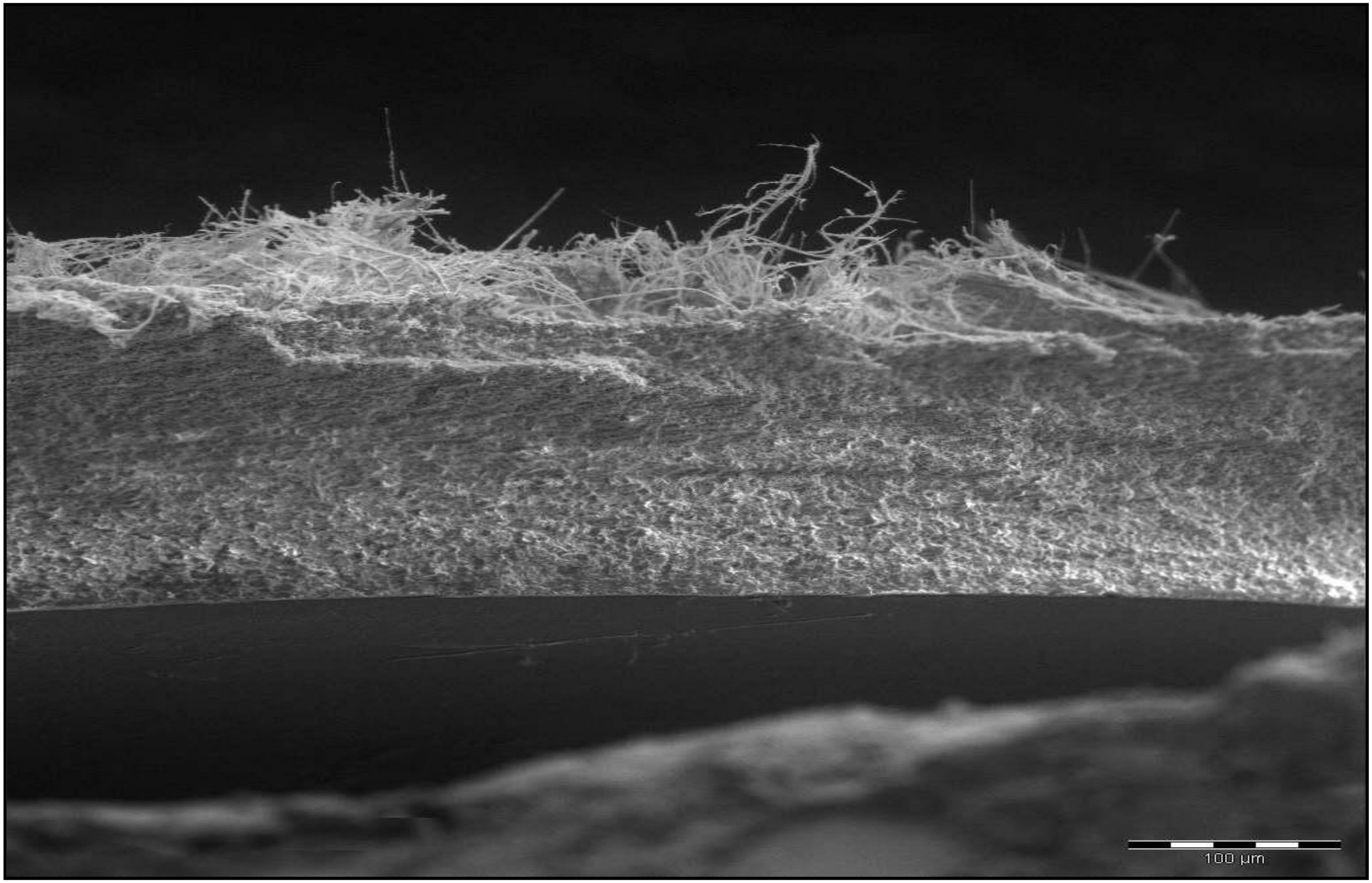

Figure 4.15: SEM micrograph of inner eggshell layer fragment of Nile crocodile. The smooth surface is directed towards the embryo, and the fibrous-appearing surface is directed towards the outer shell layer.

4.3.2. Inorganic elements and eggshell thickness

4.3.2.1 Elements in the eggshells

The possible effects of inorganic elements on the thickness of the crocodile eggshells were investigated using multivariate statistics. A series of PCAs were done using the absolute concentration data (not relativised) of the individual eggshell measurements for both the inner and outer shell layers, and the concentrations of elements found in the eggshells (Fig. 4.16-18).

The red vector in Fig. 4.16 indicates the outer shell thickness. The inner layer vector was not visible in this dimension. Note that this figure is very much the same as Fig. 4.6 (p. 60), but with the vector added. The clustering and association patterns therefore remain the same. Most of the eggs from the KNP were associated with a thicker outer shell, except for C11 (which was associated with a thinner outer shell), C15 (which was associated with no effect on the thickness of the outer shell), and C26 (which ordinated away from the rest of the eggs; Fig. 4.16). Most of the farmed crocodile eggs ordinated to the left of the origin, and therefore had thinner outer shells. Compounds negatively associated with the outer shell vector seemed mainly to have been U, and possibly Cd and Sn. Pd, Pt and Rh were only weakly positively associated.

The inner and outer shell layer vectors were both visible in the dimensions presented by axis 3 and 1 (Fig. 4.17). The two vectors (inner and outer shell thickness) are separated by about 30°. The eggs from the commercial farm (except for C3) were generally associated with thinner inner and outer shells, while the eggs from the KNP were associated with thicker shells (except for C11 and C26). C1- 4, and C8, C11, and C26 had no clear association with either vector. The only element that was associated with either shell layer vector was U - negatively for both vectors.

To further investigate the possible effects of inorganic elements on the thickness of either the inner or outer (or both) eggshell layers, a PCA was done using the ratio of the outer and inner shell layer thickness, or the layer ratio (Fig. 4.18). The layer ratio (LR) is indicated by the red vector in Fig. 4.18. The ordination is the same as in Fig. 4.16, and the vector is almost the same as for the outer layer vector as well. The same PCA is presented using axis 1 and 3 (Fig. 4.19). The ordination is almost the same as

Fig. 4.17. U was not associated with the LR vector, and Cd, Sn, Ag, Au, and Pb was associated with a negative association with the LR ratio. A ratio approach was thus not able to provide more information on the possible effect of inorganic elements in the eggshells on the thickness of the eggshells.

The clustering of the individual eggs with their specific nest sites might be a result of one or a combination of the following:

- The elements in the sand surrounding the eggs in their specific nests were absorbed by the eggshells providing the nest-specific elemental profiles that clusters some of the nests,
- The regional differences in the diets of crocodiles affected the elemental profiles in the females, and therefore the elemental profiles of the shells themselves ,
- The age of the embryo in the egg affected the concentrations of elements still present in the shells, and not extracted by the embryo.

4.3.2.2 Elements in the egg contents

In order to explore which of the above three possibilities were in effect, PCA ordinations were done, using the same variables for inner layer thickness, outer layer thickness and LR, but with the concentrations of elements in the egg contents (Figs. 4.20-21). The reasoning here is that if the elemental profiles of the eggshell and egg contents ordinate in the same way, the elemental profiles would most likely be due to maternal elemental profile, and not the ambient sand or embryo age.

There was a much less distinct clustering of eggs with their nesting origin, based on the elemental profiles of the nest contents (Fig. 20-21). The separation between the eggs from the farm and those from the KNP was also less clear when compared with the elemental profiles of the eggshells. The vectors indicating the inner and outer shell layer thickness and LR were not visible in any of the three two-dimensional aspects, even after the vector scaling was increased to 240% (Figs. 4.20-21). As a result, none of the elements could be associated with a thinning or thickening effect on either the outer or inner shell layers or both. This approach did thus not help in explaining which of the possibilities were in effect.

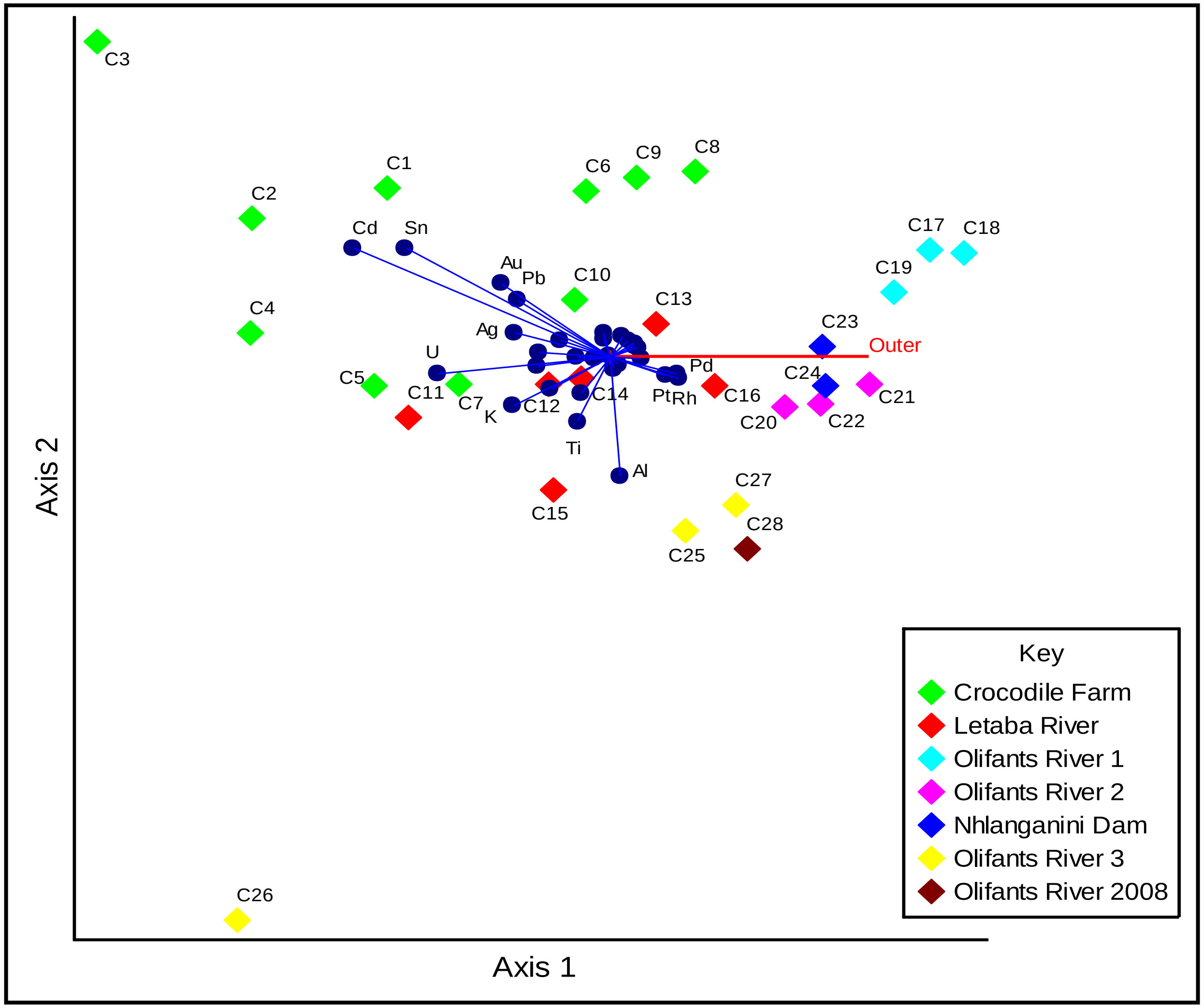

Figure 4.16: PCA plot for the possible association of inorganic elements in the eggshells with eggshell thickness. The red lines are the vectors for inner and outer shell thickness, increasing in effect away from the origin. Not all elements are labelled due to clustering. C1-C28 indicates individual eggs. Axis 1 = 8.72%, axis 2 = 5.01%, axis 3 = 3.27%, cumulative = 17%. $P < 0.05$.

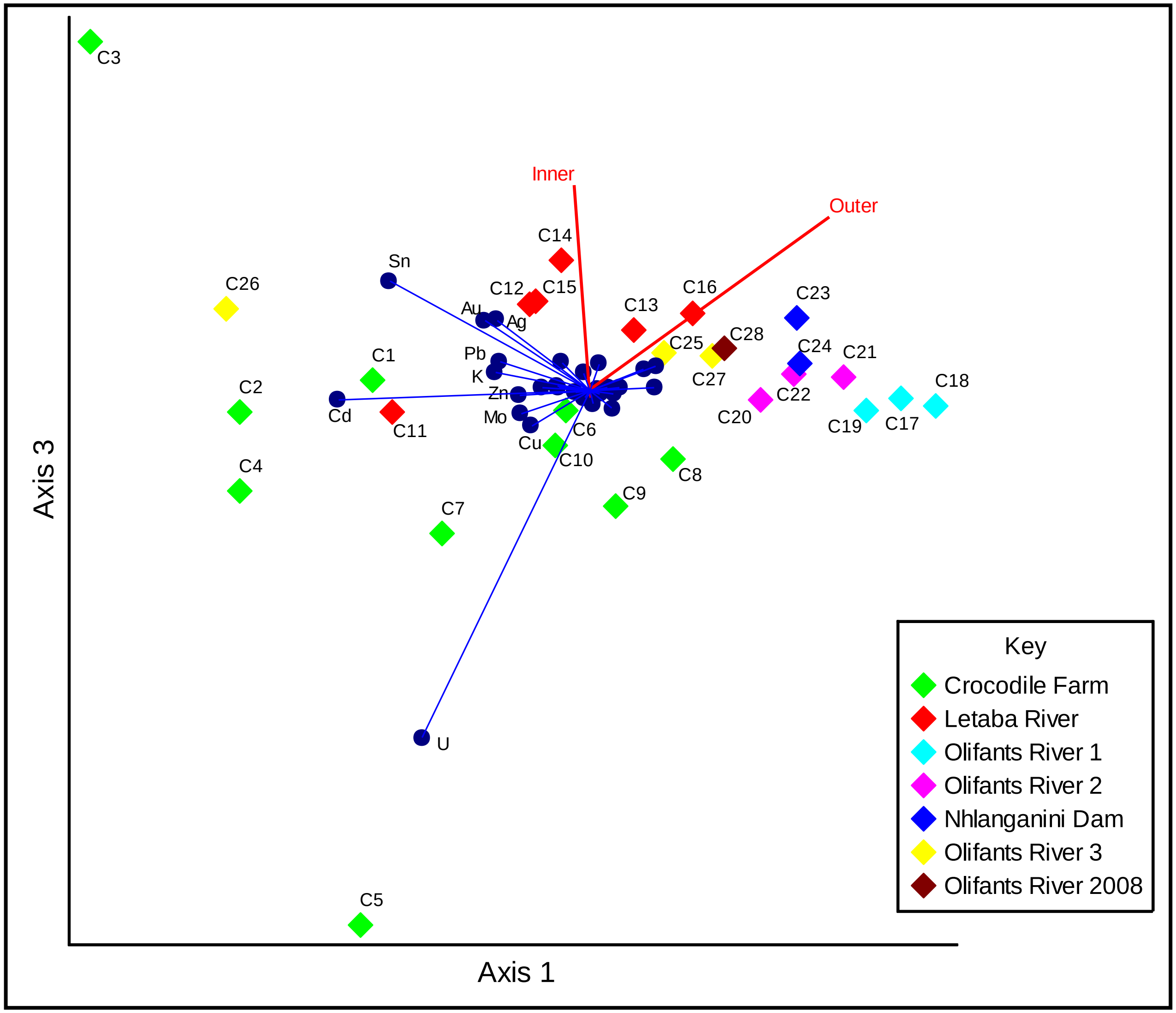

Figure 4.17: PCA plot for possible association of inorganic elements (found in the eggshells) with Nile crocodile eggshells. Axis 1 and 3 are viewed to show both inner and outer shell layers simultaneously. C1-C28 indicates individual eggs. Axis 1 = 8.72%, axis 2 = 5.01%, axis 3 = 3.27%, cumulative = 17%. $p < 0.05$.

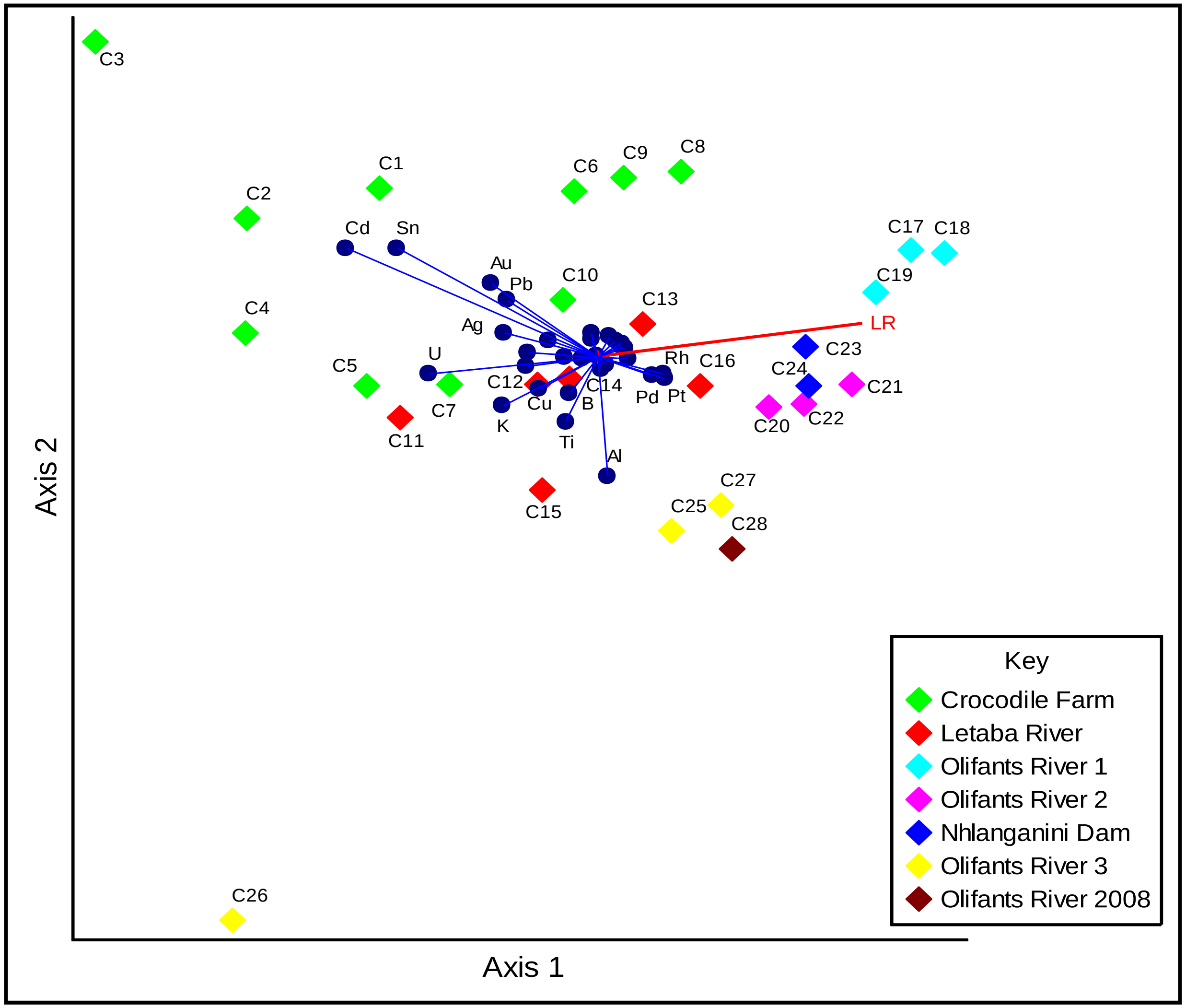

Figure 4.18: PCA plot for the possible association of inorganic elements in the eggshells with the ratio of the two layers of Nile crocodile eggs. The red line indicates the vector of the LR. Not all elements are labelled due to clustering. C1-C28 indicates individual eggs. Axis 1 = 8.72%, axis 2 = 5.01%, axis 3 = 3.27%, cumulative = 17%. $p < 0.05$.

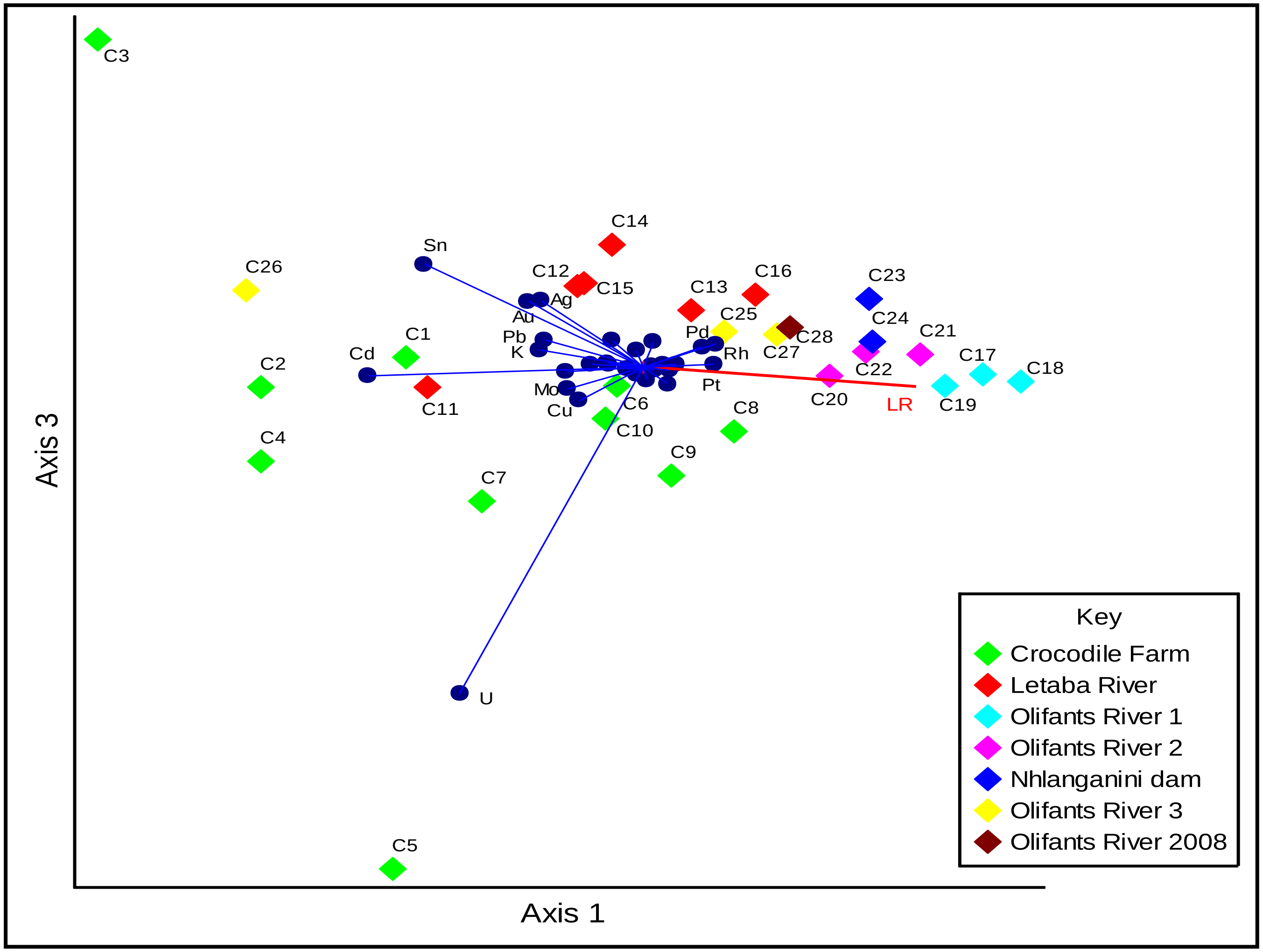

Figure 4.19: PCA plot for the possible association of inorganic elements in the eggshells with the ratio of the two layers of Nile crocodile eggs. Axis 1 and 3 are viewed. The red line indicates the vector of the LR. Not all elements are labelled due to clustering. C1-C28 indicates individual eggs. Axis 1 = 8.72%, axis 2 = 5.01%, axis 3 = 3.27%, cumulative = 17%. $p < 0.05$.

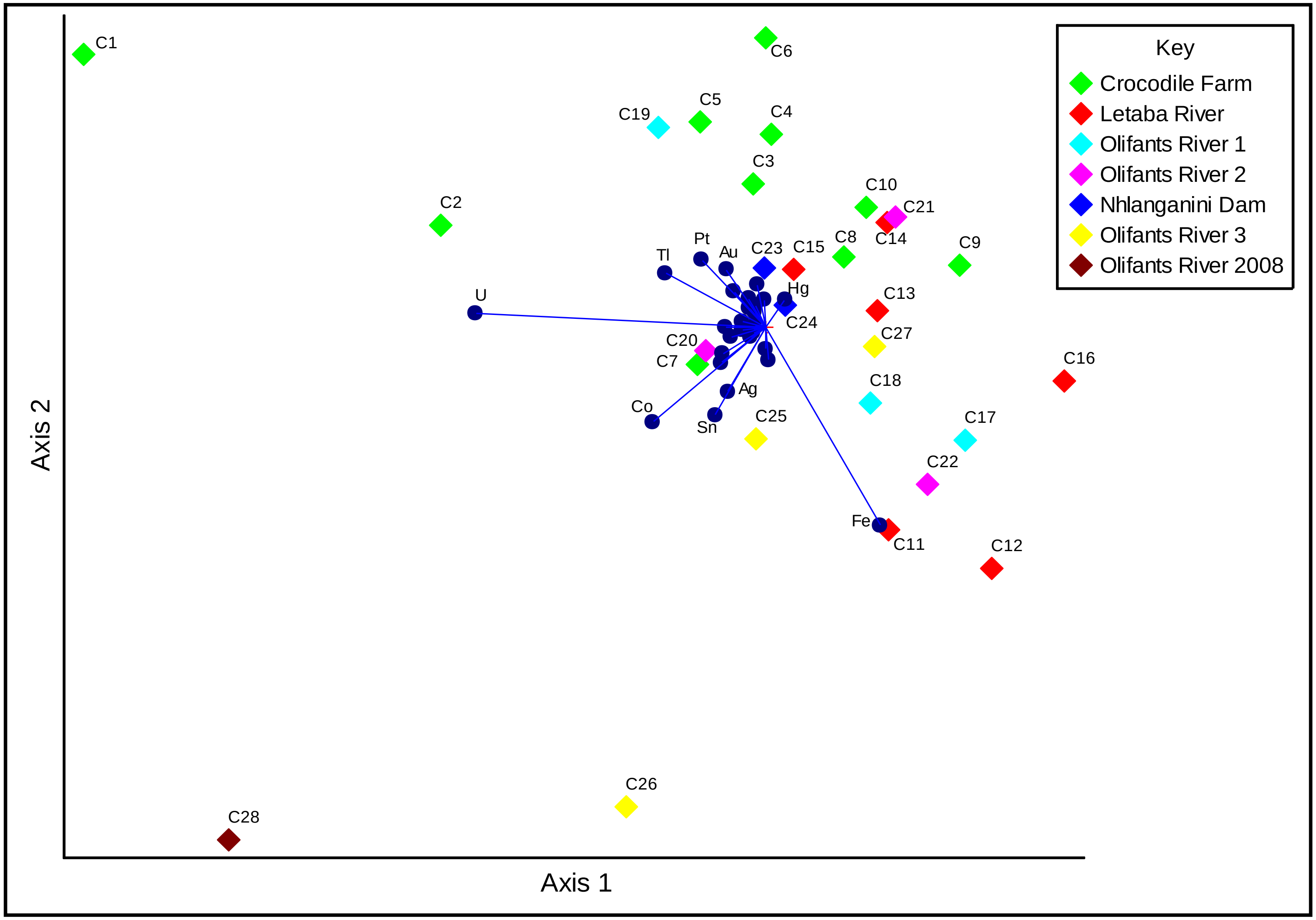

Figure 4.20: PCA plot for the possible association of elements in the egg contents with the inner and outer shell layer thickness' of Nile crocodile eggs. The vectors for the inner and outer shell thickness are not visible. Not all elements are labelled due to clustering.C1-C28 indicates individual eggs. Axis 1 = 5.35%, axis 2 = 4.47%, axis 3 = 2.93%, cumulative = 12.75%. $p < 0.05$.

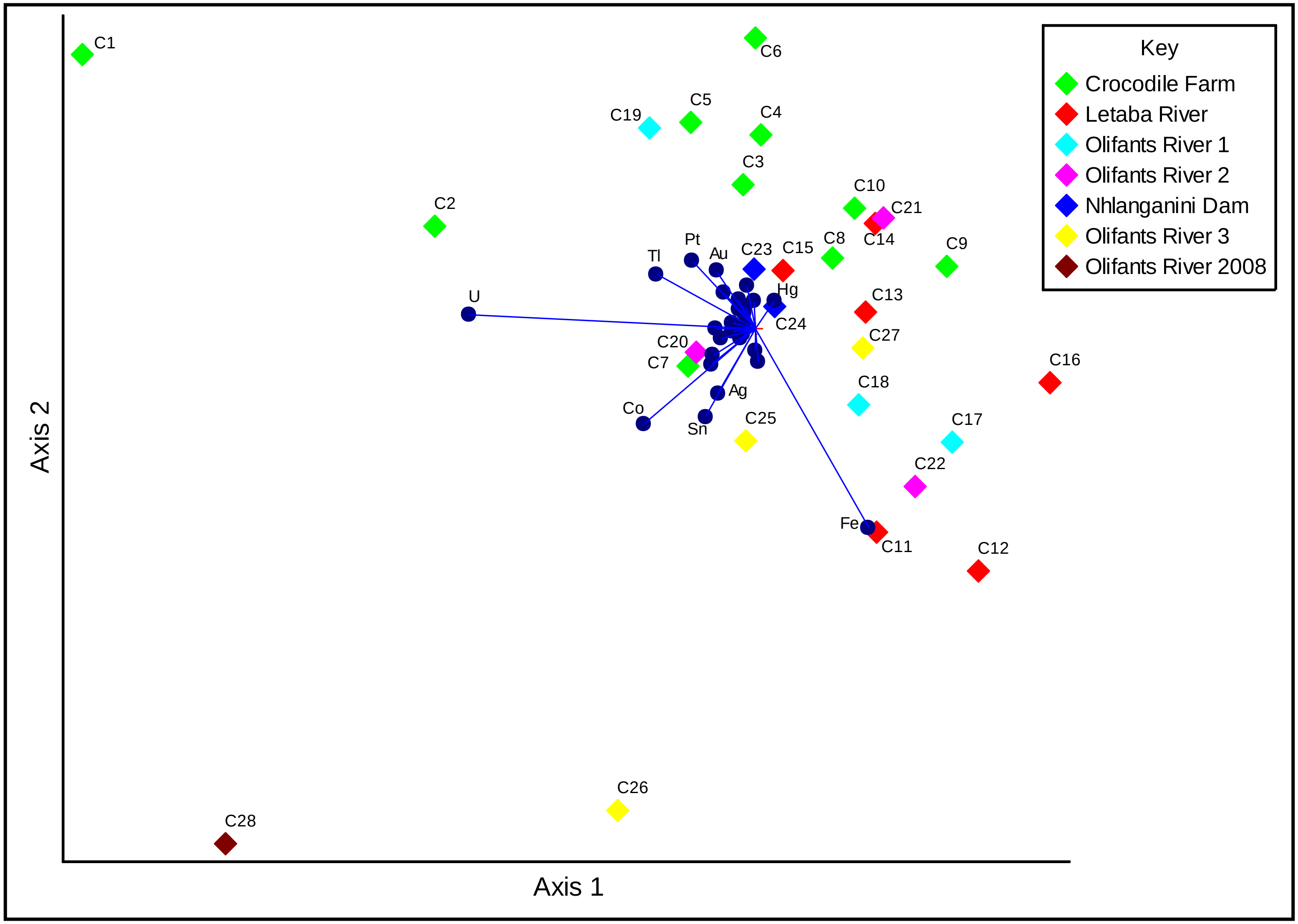

Figure 4.21: PCA plot for the possible association of elements in the egg contents with the ratio of the two layers (layer ration or LR) of Nile crocodile eggs. The vector of the LR is not visible. Not all elements are labelled due to clustering. C1-C28 indicates individual eggs. Axis 1 = 5.35%, axis 2 = 4.47%, axis 3 = 2.93%, cumulative = 12.75%. $p < 0.05$.

4.3.3. Organic pollutants and eggshell thickness

Since it is known that compounds such as DDE can affect the thickness of eggshells of birds (Lincer, 1975), such a possibility was investigated in the present case. A series of investigative PCAs were done to explore any association between the concentrations of compounds present and eggshell parameters, using absolute data (not relativised) and the mean eggshell thickness for each pool. This exploration will follow a logical progression whereby variables will be manipulated and/or eliminated based on the results and deliberation of the preceding PCAs.

In Fig. 4.22, the two red vectors (inner shell and outer shell thickness) are aligned in opposite directions. PCB-194, -180, and -156, BDE -154, -153, and -47, and all three metabolites of DDT are aligned in the direction of the inner shell vector, and opposite to the outer shell vector. PCB-199 and -128, BDE-99, and PBEB had no association with either vector. Pool 2 was associated strongly with PBEB, but with no association with either vector. Pool 3 was associated with PCB -199, also with no association with either vector. Pools 1 and 4 were associated with a thicker outer shell (or thinner inner shell), and with low concentrations of PCB-157, BDE-153, *p,p*'-DDD, p,p'-DDE, and p,p'-DDT. Pool 5 was associated with a thicker inner shell. The opposing inner and outer shell vectors could imply one or more of the following for the eggshell thickness of Pools 5, 4 and 1:

- Compounds aligned in the direction of the inner shell vector had a thickening effect on the inner shell, but not the outer shell,
- Compounds aligned in the direction of the inner shell had a thinning effect on the outer shell, but not the inner shell,
- Compounds associated with the inner shell vector had a thickening effect greater on the outer than a thinning effect on the inner shell, or
- Compounds associated with the inner shell vector had a thinning effect greater on the inner shell than on the outer shell.

To investigate which possibility is more probable, a PCA ordination was done using the LR (Fig. 4.23). The layer ratio is indicated again by the red vector in Fig. 4.23. This ordination was exactly the same as in Fig. 4.19, but with only one vector. A ratio approach did therefore not provide more information regarding the possible effects of organic pollutants on eggshell parameters of the five pools.

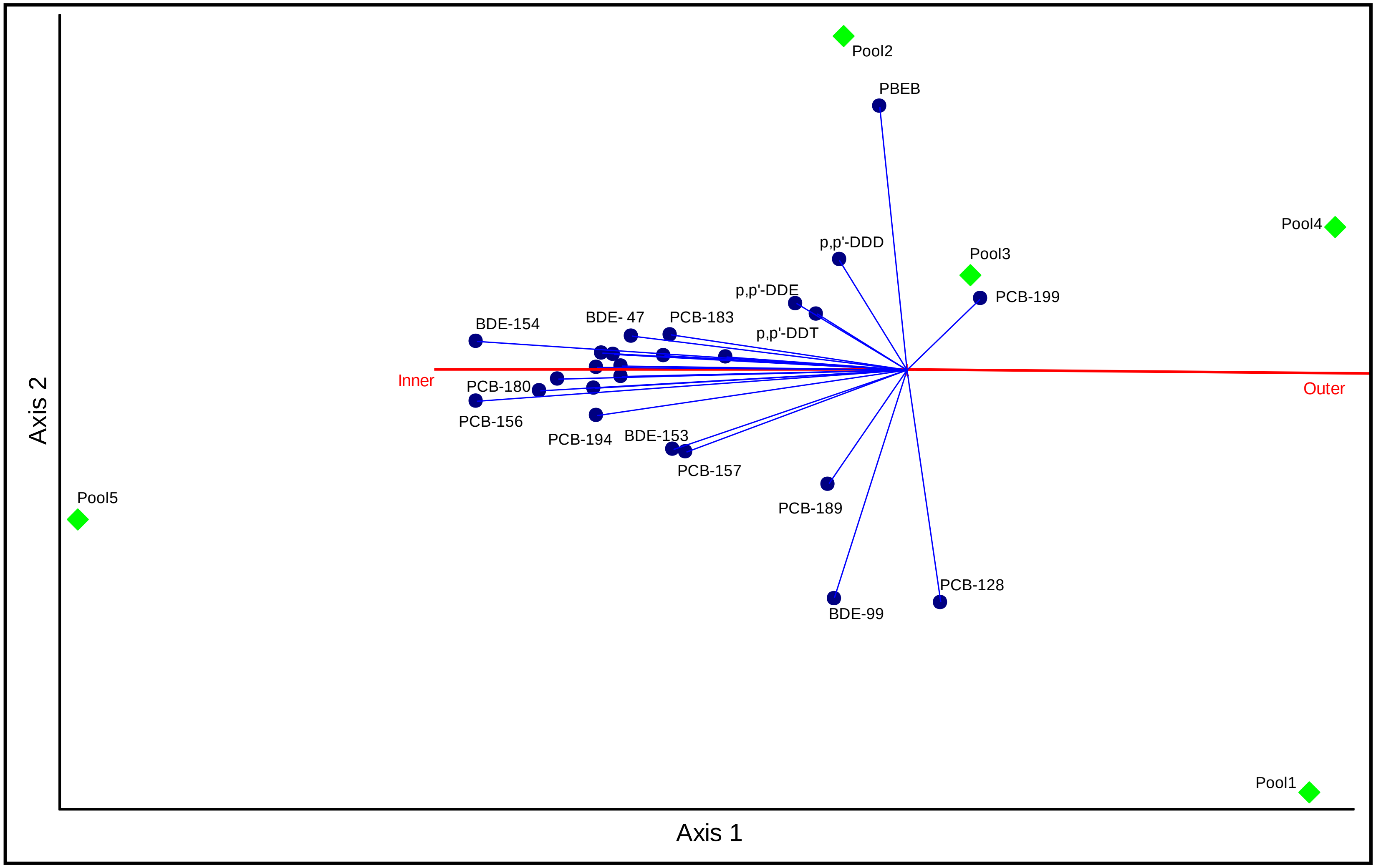

Figure 4.22: PCA plot for the possible association of organic pollutants with Nile crocodile eggshell thickness. The red lines are the vectors for inner and outer shell thickness. Not all PCBs are labelled due to clustering. Some compound labels were omitted for legibility. Pool 1 = Crocodile farm; Pool 2 = Letaba River; Pool 3 = Olifants River; Pool 4 = Nhlanganini Dam; Pool 5 = Olifants River 2008. Axis 1 = 16.41%, axis 2 = 5.34%, axis 3 = 2.76%, cumulative = 24.51%. $P < 0.05$.

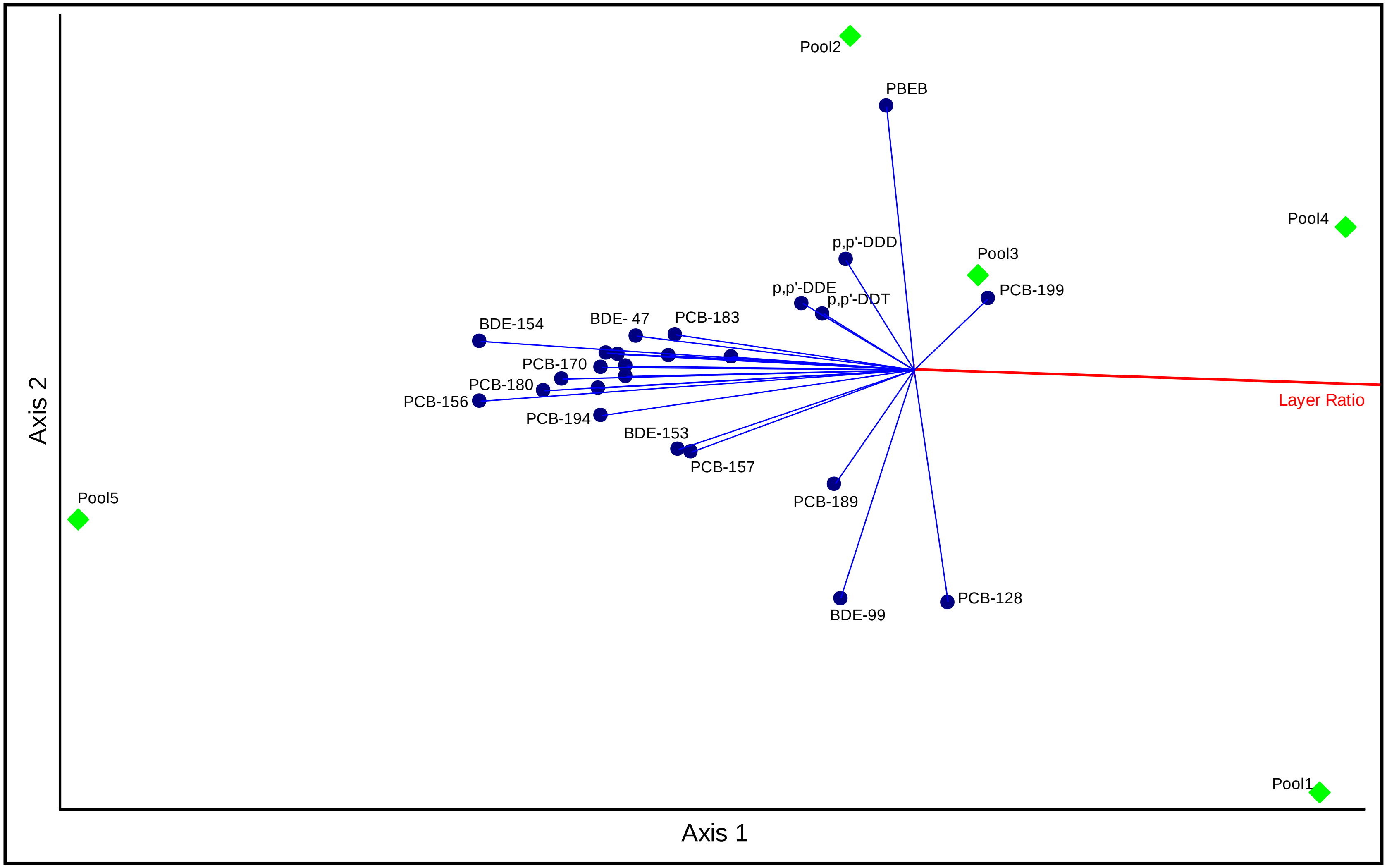

Fig 4.23: PCA plot for the possible association of organic pollutants with the ratio of the two layers of Nile crocodile eggs. The red line indicates the vector of the LR. Not all PCBs are labelled due to clustering. Some compound labels were omitted for legibility. Pool 1 = Crocodile farm; Pool 2 = Letaba River; Pool 3 = Olifants River; Pool 4 = Nhlanganini Dam. Pool 5 = Olifants River 2008. Axis 1 = 16.41%, axis 2 = 5.34%, axis 3 = 2.76%, cumulative = 24.51%. $P < 0.05$.

Because Pool 5 consisted of only one egg that was collected during a different breeding season and had higher levels of compounds overall (Figs. 4.8-11), it was not considered in the next set of analysis. The higher level of organic pollution present may distort the picture, especially as it represented only one egg. Without the Pool 5 egg, the layer vectors were now almost perpendicular, with most of the compounds aligned with the outer shell vector (either positively or negatively), which was still aligned positively with Pools 1 and 4 (thicker outer shells) and negatively with Pools 2 and 3 (thinner outer shells) (Fig. 4.24). Only *p,p'*-DDE, and PCB-199 were associated with a thicker inner shell (probably due to Pool 4 influence), and or BDE-153 and PCB-157 with a thinner inner shell. Both Pools 1 and 4 represent reference eggs. Ordinating with the LR in Fig. 4.25 shows the vector positively associated with BDE-99, and PCB-128, and negatively with *p,p'*-DDD and *p,p'*-DDE.

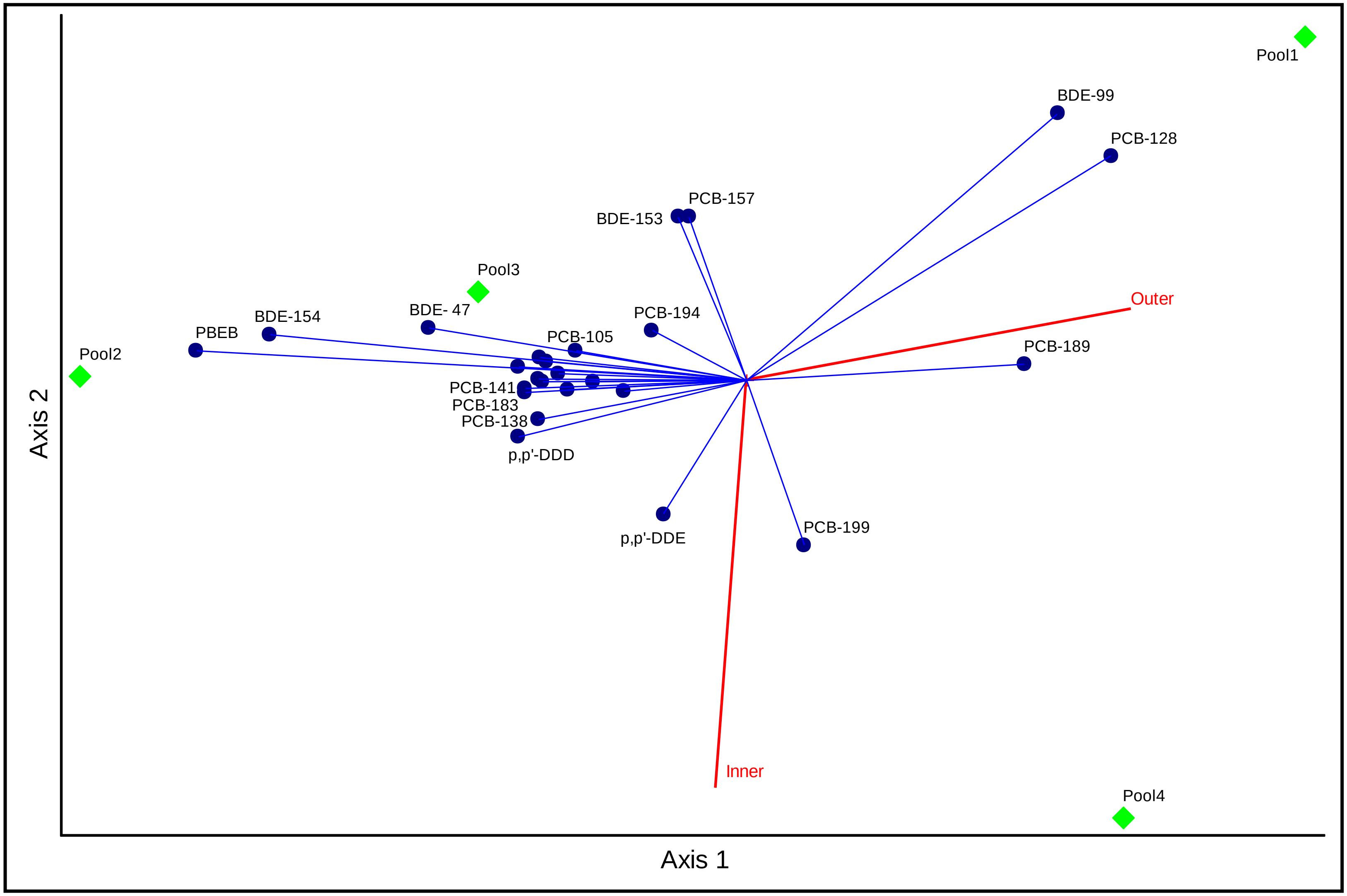

Figure 4.24: PCA plot for possible effect of certain organic pollutants on the inner and outer shell layers of Nile crocodile eggs. Pool5 was excluded from this ordination. Some compound labels were omitted for legibility. Pool 1 = Crocodile farm; Pool 2 = Letaba River; Pool 3 = Olifants River; Pool 4 = Nhlanganini Dam. Axis 1 = 18.1%, axis 2 = 5.86%, axis 3 = 1.04%, cumulative = 25.01%. $P < 0.05$.

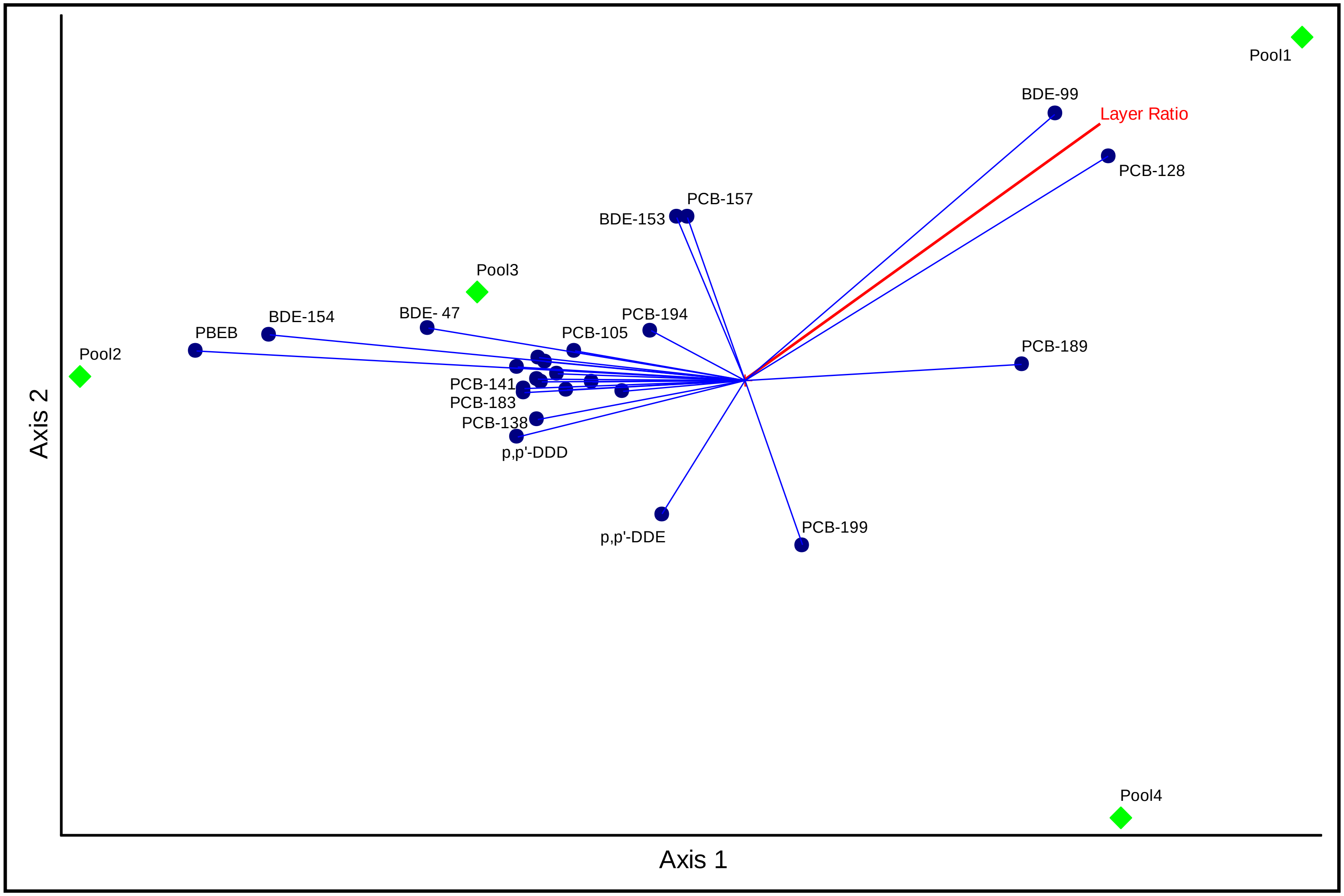

Figure 4.25: PCA plot for possible effect of certain organic pollutants on the ratio of the inner and outer shell thickness of Nile crocodile eggs. Pool 5 was excluded from this ordination. Some compound labels were omitted for legibility. Pool 1 = Crocodile farm; Pool 2 = Letaba River; Pool 3 = Olifants River; Pool 4 = Nhlanganini Dam. Axis 1 = 18.1%, axis 2 = 5.86%, axis 3 = cumulative = 23.97%. P < 0.05.

5. Discussion

5.1. Metals and other elements

5.1.1. Egg contents

Of the elements found in the contents of the eggs, significant differences were found between four of the five pools for B, Mg, Fe, Cu, As, Mo, and Ba (Table 4.3).

B concentration was the highest in the eggs from the crocodile farm (Table 4.2). Although the concentrations were low (representative species of fish and amphibians tolerate up to 10 mg/kg; Eisler, 1990a), it should be worrying as B has been found to have adverse reproductive effects on mallard (*Anas platyrhynchos*; Smith & Anders, 2009) and can be phytotoxic to other aquatic species (Eisler, 1990a).

Mg was found in higher concentrations in the eggs from the farm, the Olifants River, and the egg collected in 2008. High Mg concentrations have not been recorded as an element which can cause adverse effects in crocodile eggs. However, deficiencies in Mg is known to cause adverse effects in livestock (Hurley *et al.,* 1990), but further studies on crocodilians need to confirm the possible effects on the eggs (and reproduction).

Although the concentrations of As in Nile crocodile eggs were low, it was higher than the concentrations found in American alligator eggs from polluted lakes (Table 4.4). The concentrations of As were the highest in the eggs from the commercial farm (Table 4.4).

It was not surprising that Hg was found in crocodile eggs as it is readily found in aquatic systems (Berlin *et al.,* 2007). There was no difference between the concentrations of Hg in the eggs from the commercial farm and the eggs from the KNP. Surprisingly though is that the concentrations of Hg found in the eggs during this study were lower than the concentration in the eggs from the literature cited (Table 4.4).

Hg and As can be highly toxic, but it needs to be present in high concentrations (Fowler *et al*., 2007; Berlin *et al*., 2007). The LD_{50} of Hg and As for Nile crocodile embryos are not known.

Mo concentrations were difficult to compare with reported levels, as the level of 0 mg/kg dw was probably also the LOQ for that study on American Alligators (Table 4.4). The highest concentrations of Mo were found in the Nhlanganini Dam, the commercial farm, and the egg collected in 2008 (Table 4.2).

Of the five pools in this study, Ba concentrations in the egg contents was the highest in the egg collected in 2008 (Table 4.2). It is possible that the concentration of Ba in this egg would be closer to the concentrations of the other pools, if it consisted of more than one egg.

The Fe concentrations in the egg contents were much higher than the water quality guidelines for Fe in British Columbia (1mg/kg total iron; Water Stewardship Division, 2008). The highest concentration of Fe was found in the egg collected in 2008.

In the PCAs of the elemental concentrations in the egg contents, there was little separation between the eggs from the farm and those from the KNP, although the MRPP for these two groups showed a significant difference ($p < 0.05$). There was also no separation, or individual clustering by respective nests, which is supported by the MRPP for the six groups ($p > 0.05$; Fig. 4.3 and 4.4). It is important to keep in mind that the eggs from the crocodile farm were not from the same individual female, but randomly selected from the breeding chambers. Also important is that the eggs from the crocodile farm were not laid by females of the same age, as the sizes of the eggs differed (Thorbjarnarson, 1996). It is possible that higher concentrations of elements would be present in the older females, as time allowed for elements to accumulate more. These higher concentrations of elements would affect the elemental profile of the females and the eggs, and would thus explain the wide ordination of the farm eggs in Fig. 4.3. This figure also showed that the elemental profiles of the eggs from the KNP and those from the farm did not differ very much. The breeding crocodiles from the commercial farm were farm-reared animals. The strong association of U to C16 (Fig. 4.3) could not be explained, as the eggs from this nest (on the Letaba River) had the lowest mean concentration of U (Table 4.2).

5.1.2. Eggshells

The chemical composition of the Nile crocodile eggshell is not well known, but it is presumed to be similar to that of American alligators (Ferguson, 1985, and see Section 2.3.6.6). Of the elements found in the eggshells in this study, significant differences were found between four of the five pools for B, Na, Cr, As, Mo, Rh, Pd, Ba, and Hg (Table 4.3).

The B concentrations in the eggshells were the highest in the eggs from the Letaba River (Table 4.5). The concentrations in the Letaba River eggshells were higher than the concentrations found in the egg contents of any pool (Table 4.2). The elevated B concentrations in the Letaba River suggest an abnormal high concentration in the shells from this site.

The Na concentrations in the eggshells were much lower than in the egg contents (Tables 4.2 and 4.5) which is odd considering that Na is one of the components of the crocodilian eggshell (Ferguson, 1985). It is possible that Na is extracted by the developing embryo, along with Ca, which would explain the higher concentrations in the contents than in the eggshells (Tables 4.2 and 4.5). The highest concentration of Na was found in the egg contents from the farm (Table 4.2).

The lowest concentrations of Cr in the eggshells were found in the eggs from the farm. The chromium concentrations in the egg contents and shells from this study was lower than the concentration found in the contents and eggs from the literature cited (Tables 4.4 and 4.7).

The As concentrations in the eggshells for this study showed the lowest concentrations in the farm eggs (Fig. 4.5). In contrast, As was found in the highest concentration in the contents of the farm eggs (Table 4.2). The As concentrations in the farm eggs were higher in the shells than in the content.

The highest concentration of Mo in the eggshells was also found in the farm eggs (Table 4.5). The concentration of Mo was evenly distributed between the eggshell and contents (Tables 4.2 and 4.5).

The concentrations of two platinum group metals (Pd and Rh) were higher in the eggshells from the Olifants River, the Letaba River, and the Nhlanganini Dam, than in the farm eggs (Table 4.5). These two elements are among many that are extracted by platinum mining activities such as found in the Olifants River Catchment.

Hg concentrations in the eggshells were the highest in the eggs from the Letaba River and the farm (Table 4.5). This is in accordance with the Hg concentrations found in the egg content (Table 4.2). Hg concentrations were lower in the eggshell than in the egg contents (Tables 4.2 and 4.5). The relatively low concentrations of Hg in the eggshells and contents could possibly be a result of the ability of animals to excrete Hg (Berlin *et al.,* 2007).

The Ba concentrations in the eggshells were lower in the eggs from the Olifants compared with eggs from the other pools (Table 4.5). The concentrations of Ba in the shells of the eggs from this study were lower than in the content of the eggs (Table 4.2).

Hg, Cr, and As concentration in the eggshell and egg contents from this study were lower than the concentrations found in eggshells reported by other studies (Table 4.7). The Mo concentrations found in the egg contents from this study was higher than the concentrations found in the literature cited (Tables 4.4 and 4.7).

According to Table 4.2 and Table 4.5 some elements (such as Mg, Cr, Ba) are found in higher concentrations in the shells than in the contents, and some elements (such as B, Na, As) are found in higher concentrations in the contents of the eggs. The embryo extracts calcium from the shell during development (Ferguson, 1985), and the possibility of other metals also being extracted at the same time could be further investigated.

The concentrations of elements found in the shells resulted in a much closer clustering of the eggs to their respective nest sites in the multivariate analysis (Fig. 4.6). The separation between the nests was supported by the MRPP ($p < 0.05$). There is also a clear separation between the eggs from the crocodile farm, and those from the KNP (Fig. 4.7). The separation between the eggs from the KNP and those from the farm was also supported by the MRPP for the two groups ($p < 0.05$).

5.1.3 Possible sources

The overall concentrations of elements found in the shell and contents of the eggs from the KNP are relatively low when compared with the reference sites and other studies (Tables 4.4 and 4.7). The farm eggs generally had the higher concentrations of elements (Tables 4.2 and 4.5). With such low concentrations, it is possible that the sources of many of these elements (such as B and Ba) are natural and not anthropogenic. However, naturally occurring ($BaSO_4$) is not absorbed (Oskarsson & Reeves, 2007). Thus, an unknown proportion of the elements present most likely entered the environment anthropogenically. The ways through which different elements are released into the environment *inter alia* include:

- Mining or industrial activities with associated discharge of elevated elemental concentrations into the water, air and soil (Mo, Ba, Cr, Rh, and Pd; Turnlund & Friberg, 2007; Osskarson & Reeves, 2007; Barbante *et al.,* 2001),
- Livestock farming; introducing elements such as Mo into the environment through run-off during the rainy seasons (IWMI, 2007; RHP, 2001a).

Although it is possible that all the elements in the eggshells and egg contents were entirely derived from maternal transfer (Rainwater *et al.,* 2002), the variation in concentrations in the eggshells and especially in the egg contents implies that a proportion of the elements were derived from the ambient sand in which they were laid, as well from the females during egg formation.

The clustering of the individual eggs with their respective nest sites in the ordinations (Figs. 4.16-4.19) might be a result of one, or a combination of the following:

- The elements in the sand surrounding the eggs in their specific nests were absorbed by the eggshells providing the nest-specific elemental profiles that clusters some of the nests,
- The age of the embryo in the egg affected the concentrations of elements still present in the shells, and not extracted by the embryo. The difference in the age of the eggs may have affected the concentrations of elements extracted from the eggshells by the embryos,

- The regional differences in the diets of crocodiles affected the elemental profiles in the females, and therefore the elemental profiles of the shells themselves. Although all eggs are laid by the female in one clutch, the eggs do not develop at the same rate in the female, as shown in Fig. 5.1. There are many small and large eggs developing, but without their shells. The shell formation is probably simultaneous and just before egg deposition commences. It is possible that due to differential development of the eggs, the elemental profile will differ between individual egg contents as the stores of these elements in the female become depleted (possibly attenuated by feeding during the egg development). Once all eggs have developed, and during eggshell formation (presumably all eggs at once), the shells may much more resemble each other in terms of elemental profile than the egg contents that developed prior to shell formation and at different rates.

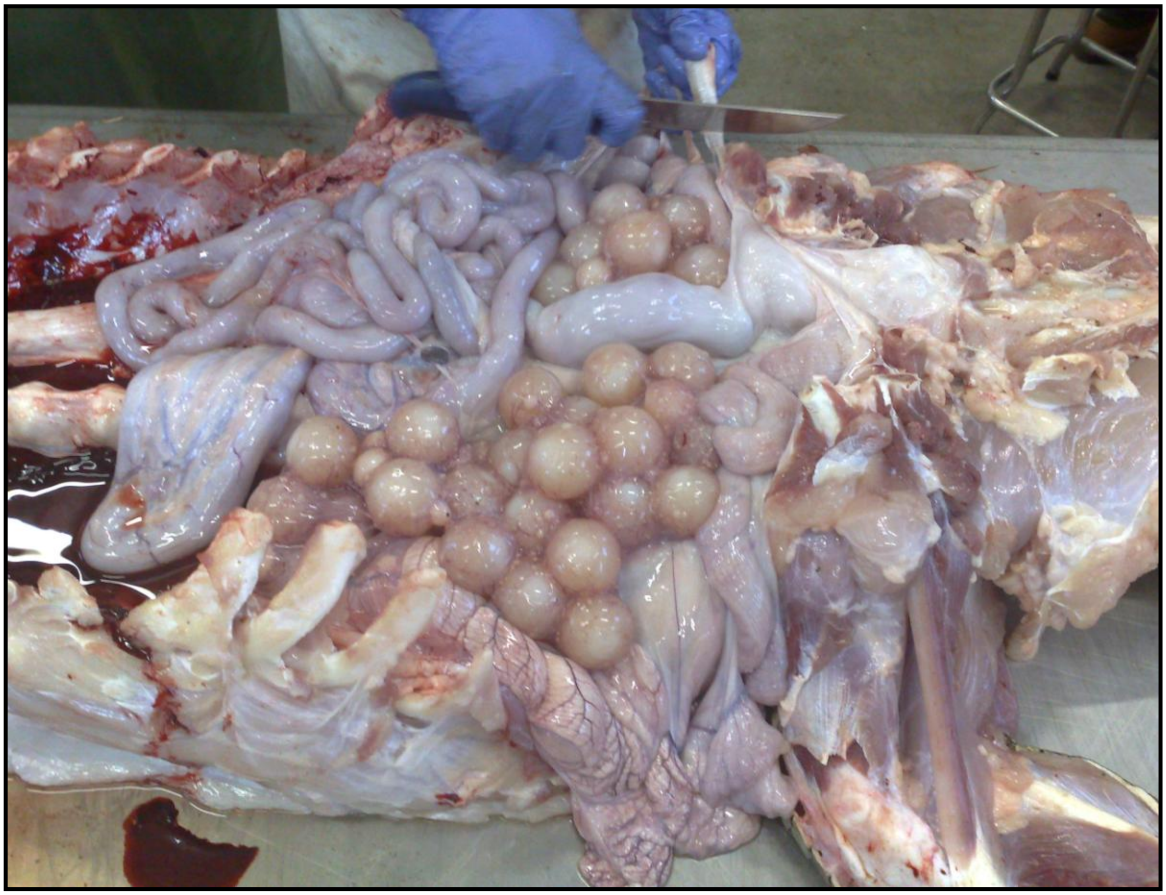

Figure 5.1: Photograph of the abdominal area of a female Nile crocodile. The photograph illustrates the different developmental phases of the eggs in the female.

Considering the results discussed thus far, the most probable source of the elements in the eggs (content and shells) is a combination of maternal transfer during egg formation, and the ambient sand of the nests. It is unlikely that the developmental phase of the embryos differed enough to influence the elemental profile of the egg content and eggshells, as the eggs were collected over a short period at the beginning of the Nile crocodile breeding season. The most likely route of uptake of these elements by the females is through their prey (or food in the case of the farmed crocodiles), and not via drinking water or respiration. The uptake of these elements resulted in maternal transfer during egg formation. This is also the most probable method of transfer for organochlorines in crocodilians (Wu *et al.,* 2000). The difference in the elemental concentrations between the farmed and wild crocodiles is therefore likely due to differences in diets. The wild crocodiles rely on a wide variety of aquatic and terrestrial organisms that they catch (Pooley, 1989) which would represent the elemental profiles the prey come into contact with. The farmed crocodiles on the other hand are fed mainly poultry collected from batteries in the Highveld region. It is possible that the chickens are contaminated with higher concentrations of elements, either through their feed, or through contaminated water or air.

The differences between eggs from the same clutch could not be explained. However, of the three possibilities listed in bullets above, the last explanation seems quite convincing, as it considers elemental profiles of both egg contents and shells. However, the effects from the sand cannot be discarded and needs further investigation.

5.2. Organic pollutants

5.2.1. Organochlorines in egg contents

The highest concentration for almost all PCBs were found in the egg from the Olifants River collected in 2008 (Fig. 4.8). The major PCB congers were PCB-138, 153, 170, 180, 183, and 187 (Fig. 4.8), although almost all PCBs analyzed for were found

Of the non-DDT pesticides analyzed, β-HCH was the major conger in the egg contents (Fig. 4.9). HCH has been used as a pesticide since the 1940's (Breivik *et al.,* 1999). HCH was only added to the Stockholm convention in 2009 (annex A), which is possibly why it is still likely to be present in the environment due to past use as insecticidal agent (SC, 2011).

HCB was also found in high concentrations in the crocodile eggs (Fig. 4.9). HCB has strong toxicological effects on biota (Bailey, 2001), and may pose a threat to developing crocodile embryos (Alvarez *et al*., 2000). However, the effects levels are not known.

The chlordane concentrations in the content of the eggs were very low (Fig. 4.9) compared with the concentrations of chlordane in eggs of American alligators (Heinz *et al.,* 1989) and American crocodiles (Hall *et al.,* 1979). The low concentrations in the Nile crocodile eggs from this study may indicate that levels of chlordane in the sampled areas have decreased since agricultural use was ceased in 2000 in SA (Batterman, 2008).

The presence of Mirex in the egg contents was surprising (Fig. 4.9) as it has never been registered in South Africa as a pesticide. The reduction in the concentration of mirex in crocodile eggs of 2008 to 2009 (Fig. 4.9) could not be explained, but is most likely linked to the reductions of the other organic pollutants during the same period.

Of all the metabolites of DDT, *p, p'*-DDE was found in the highest concentrations in all pools (Fig. 4.10). The lowest concentration was in the eggs from the commercial farm. Surprisingly perhaps was the *p, p'*-DDE in the eggs from the Nhlanganini Dam (Pool 4) that also had the highest ∑DDT of all the eggs from the KNP (except the 2008 egg). This possibility is further strengthened when looking at the elemental profile of the Nhlanganini Dam which was the same as for the other KNP eggs (Fig 4.6).

The chickens fed to the farm crocodiles originate from a chicken farm in the Highveld area. DDT is still being used in South Africa for malaria control in some rural areas (Bouwman *et al.,* 2006), but not on the Highveld. It is possible that DDT-contaminated feed was given to the chickens, thereby accumulating in the crocodiles. This needs to be established with analysis of the chicken feed.

∑Organochlorines were also higher in the eggs from the Nhlanganini Dam than in the eggs from the Olifants and Letaba rivers, but was due to the high ∑DDT concentrations (Fig 4.8-4.10). The source of the DDT is not known, but may have been due to the female being exposed to polluted prey elsewhere.

The presence of organochlorines in the eggs are worrying as these compounds have been found to have a strong negative correlation with broad-snouted caimen (*Caiman latirostris*) clutch size in a river in Argentina (Stoker *et al.,* 2011). If the organochlorines compounds have the same effect on the Nile crocodiles in the KNP, it will have a devastating effect on a already declining population.

5.2.2. Brominated flame retardants in egg contents

No literature could be found to compare concentrations of BFRs in crocodile eggs (making this the first report of such levels), but comparisons with the concentrations of organochlorines, the concentrations of BFRs in the eggs from this study seemed relatively low (Fig. 4.8-4.11). BDE-209 was found in the highest concentration in the egg collected in 2008 (Pool 5; Fig. 4.11). To our knowledge, this is also the first record of PBEB found in any crocodile egg, or any egg from Africa. PBEB is a relatively ‘new’ flame retardant, and is not on the Stockholm Convention’s list of POPs. PBEB was found only in the eggs from the Letaba and Olifants rivers, but at very low concentrations (Fig. 4.11).

5.2.3 Possible sources

The most likely sources of PCBs in the eggs from the KNP and crocodile farm are spills from combustion, high temperature processes, or leaks from transformers, hydraulic systems or capacitors (Yu, 2005). PCBs have long half-lives in soil, sediment and biological tissue (Jones & de Voogt, 1999).

The low concentrations of mirex are attributed to illegal use as pesticide or leakage from dumped products containing mirex as flame retardant. The latter case seems more likely as consistent but very low levels have been found in bird eggs from different regions from South Africa (Bouwman *et al*., 2007).

Possible sources of BFRs include sewage (BFRs are used in some pharmaceuticals; Alaee *et al*., 2003) and the dumping of products containing BFRs (de Wit, 2002). The presence of PBEB was surprising, and contamination could have occurred because of dumping of plastics and other products containing the compound (Yu, 2005).

Compounds used in pesticides or fungicides (DDT, HCB, chlordane, HCH) most likely enter the environment through air transport. The presence of *p, p'*-DDE in the eggs from the dam could possibly be attributed to transport through air (Ritter *et al.,* 1995) after the use of DDT in nearby rural areas to control malaria. However, the same levels would then be expected elsewhere in the KNP. Another, more likely possibility is that movements of the female crocodile (Magnusson *et al*., 1989) brought her into areas contaminated by DDT and chlordane, such as the Letaba River that is linked to the dam whose entire upstream catchment is within the KNP.

5.2.4 Comparisons with other data

The concentrations of some halogenated hydrocarbons (HCB, HCHs, DDTs, and PCBs) found in Nile crocodile eggs in Zimbabwe (in 1981 and 1982) were significantly higher than the concentrations found in the eggs of Nile crocodiles from this study (Tables 4.8 and Fig. 4.12). The lower concentrations in the SA crocodile eggs suggest that the environment of the Nile crocodiles from this study is much cleaner, but that supposes a very quick change in body levels when the environmental levels change.

It should also be taken into account that not all organic pollutants were analyzed. Analyzing for more compounds may influence the findings, but this was considered unlikely as other POPs and halogenated compounds would also be at similarly low levels, at least less than DDT that is still actively used. The concentrations of *p,p;*-DDE, *p,p'*-DDD, and *p,p'*-DDT in the eggs from this study was significantly lower than the concentrations found in Morelet's crocodile eggs in 2006 (Wu *et al.*, 2006). Even though the concentrations of these POPs are low in the South African crocodile eggs, it may still pose a threat to the reproduction and survival of the crocodile population. Many POPs are known endocrine disruptors (Guillette *et al.*, 1994). One of the possible hormonally controlled processes that could be disrupted by endocrine disrupting agents is the formation of the eggshell prior to laying. This is the subject of the next section.

5.3 Eggshell thickness

5.3.1. Elemental concentrations and eggshell thickness

This investigation on possible association of pollutants with eggshell thickness of the Nile crocodile is, as far as could be established, the first such investigation for crocodilians.

5.3.1.1 Elements in the eggshell

With both vectors for eggshell thickness visible in Fig. 4.17, it was clear that the eggs from the crocodile farm have associated with thinner eggshells, while the majority of the eggshells from the KNP have associated with thicker eggshells. A possible reason for the thinner eggshells of the farmed eggs could be the apparent association of the layer vectors with U and Cd that are known EDCs (Lavicoli *et al*., 2009; Raymond-Whish *et al*., 2007).

5.3.1.2. Elements in the egg contents

The vectors for the thickness of the eggshells and the LR were not apparent on the bi-plots (Figs. 4.20-21) and it is therefore unlikely that these levels influenced eggshell thickness. This is consistent with the observation that the eggshell elemental profiles may be more representative of the maternal elemental profile at the time of egg formation. It is during eggshell formation when endocrine disruption by elements would be suspected to influence eggshell thickness and not during the development of the egg.

5.3.2. Organic pollutant concentrations and eggshell thickness

Because eggshell formation is controlled by the endocrine system in birds (Romanoff & Romanoff, 1949), it is possible that the same would apply to crocodiles, because of their evolutionary ancestry (Section 2.3.2).

From the PCAs (Fig. 4.22-23) it is clear that the vectors for the inner and outer shell layers were aligned in opposite directions (Fig.4.22). Pool 5 (the single egg collected in 2008) was associated with a thinner outer shell (or a thicker inner shell), and Pool 1 (crocodile farm) and 4 (Nhlanganini Dam), both presumed reference sites, were associated with thicker outer shells (or thinner inner shell). *p,p*'-DDE, which is known to cause eggshell thinning in bird eggs (Lincer, 1975), was associated with a thinner outer shell, but a thicker inner shell.

The majority of the other halogenated organic pollutants were also associated with thicker inner shells and thinner outer shells. PCB-128, 189, and 199, PBEB, BDE -99, and *p,p*'-DDD were associated with neither shell layer vectors (Fig. 4.21).

The opposing inner and outer shell vectors could imply one or more of the following for the eggshell thickness of Pools 5, 4 and 1:

- Compounds aligned in the direction of the inner shell vector had a thickening effect on the inner shell, but not the outer shell,
- Compounds aligned in the direction of the inner shell had a thinning effect on the outer shell, but not the inner shell,
- Compounds associated with the inner shell vector had a thickening effect greater on the outer shell than a thinning effect on the inner shell, or
- Compounds associated with the inner shell vector had a thinning effect greater on the inner shell than on the outer shell,
- The pattern seen may be artefactual due to too few data.

Pool 5 was excluded from the PCAs (because it consisted of only one egg, was collected in a different breeding season, and contained high concentrations of pollutants overall) to investigate which possibility might be in effect (Fig. 4.23-24). [It is possible that the presence of Pool 5 in the PCA affected the ordination of the elements and the other pools, because it contained the highest concentrations of organic pollutants overall (Fig. 4.8-4.11).] With the Pool 5 data depleted, the vectors for the thickness of the shell layers were almost perpendicular to one another. Pool 1 (crocodile farm) was associated with a thicker outer shell. Pool 4 (Nhlanganini Dam) was associated with thicker inner and outer shells. Pool 2 (Letaba River) and Pool 3 (Olifants River) were associated with thinner outer shells (Fig. 4.23). Almost all the compounds were still associated with a thinner inner shell (Fig. 4.23), including PBEB which is not registered as a POP (SC, 2011).

The layer ratio approach was again used anticipating that it might shed more light on the possible association between organic pollutants concentrations in the egg content and eggshell thickness (Fig. 4.24). *p,p'*-DDE was associated with a smaller ratio, and therefore a relatively thinner outer shell. These two ordinations (Figs. 4.23 and 4.24) however, still did not indicate which of the first four possibilities might be in effect. It is possible that the outer shell layer was affected the most, and that Pool 2 and Pool 3 were affected the most with thinner outer shell layers. With only pooled data available, it is yet not possible to determine which of the five possibilities might be in effect. It may, however, be possible to determine which layer was affected if individual layer thickness data could be compared with egg-specific compound concentrations which were not available.

6. Conclusions

This study has shown that the elemental profiles of the egg contents and eggshells of Nile crocodiles were different. Some inorganic elements were found in higher concentrations in the eggshells than in the contents, while others were found in higher concentrations in the egg contents. Overall, the Hg, Cr, and As concentrations in the eggshells and contents were not high when compared to the concentrations found in some other studies of bird and crocodilian eggs. If these elements had had an effect on the eggs it would probably be less severe when compared with the effects on the eggs from the cited literature.

The highest concentrations of elements in the shells and contents were found in the farm eggs. A combination of the diet of the breeding crocodiles, consisting mainly of poultry, and the elemental profiles of the ambient sand of the nests, were likely reasons for the farmed eggs having higher elemental concentrations then eggs from the KNP. It was considered unlikely that the ages of the embryos differed enough to influence the elemental profiles of the egg content and eggshells, but this needs to be established.

Regarding the differences in elemental profiles in egg contents and shells, and between eggs from the same nest, it is probable that the eggs that develop at different rates in the same female will eventually have different elemental profiles as the elements gets depleted at different rates by the preceding (larger) eggs. The eggshells on the other hand is presumed to be formed all at the same time and would reflect each other in elemental profile much more than their associated contents. However, it remains possible that the ambient sand may contribute or deplete elements into or from the shell.

The ongoing use or past-use legacy of some pesticides (such as DDT and β-HCH) in South Africa was shown through their presence in all crocodile eggs. Although the Stockholm Convention has succeeded in reducing use and/or release of certain persistent organic pollutants, residues are still present in the environment.

Evidence of decreasing levels of certain organic pollutants between 2008 to 2009 breeding seasons was found. Although this was based on one egg from 2008, the difference in concentrations were large and convincing, but presumes that the levels in crocodiles change in concert with the levels in the environment. The concentrations of most of the organic pollutants found in the eggs were in all respects relatively low. That the levels of *p,p'*-DDE was higher in the eggs from the Nhlanganini Dam than the concentrations in the eggs from the Olifants and Letaba rivers was surprising and may have been due to the individual movement history of the female, rather than elevated levels of the compounds at the dam itself.

Suggestive but inconclusive associations were found between concentrations of inorganic elements in the content and shells of the eggs and eggshell thinning. Differences in egg layer thickness were associated with known ED metals such as Cd, and U, but could not be confirmed. However, the consistency in the indicative associations found here suggests cause for further investigation.

The concentrations of most of the organic pollutants in the eggs also suggest a thinning effect on the eggshells. The precise mechanisms involved, and which of the layers (inner or outer) were affected, is not certain. The eggs from the Nhlanganini Dam and from the crocodile farm (the two presumed reference sites) had thinner shells. The eggs from the Olifants and Letaba rivers had thinner shells. Because only the pooled data for the concentrations of the organic pollutants were available, further investigation on eggshell thinning was problematical and needs more data.

If eggshell thinning is in effect in the crocodiles in the KNP, either through the ED elements or the halogenated compounds or both, the small effect seen here does not immediately imply loss of eggs due to shell breakage as with bird eggs. Crocodile eggs are deposited initially with softer shells that harden after it has been covered by sand. However, if endocrine disruption is in effect in the crocodiles in the KNP, it may affect gender determination over and above the effect of ambient temperature. Since sexing of live crocodiles is impossible without capture, and crocodiles can live for many decades, a skewed sex ratio might only become apparent after many years. If the majority of the ED compounds and elements act as estrogens in crocodiles as elsewhere, it could be expected that the eventual sex ratio would shift towards females. If eventually this becomes the case (if it has not already happened over the decades of pollution in the KNP), the effects on the crocodile population will have long-term conservation and environmental consequences.

No direct connection between the concentrations of organic pollutants or inorganic elements in the eggs of the crocodiles and the seasonal mass mortalities of Nile crocodiles in the KNP could be made. The concentrations of the elements and compounds were just not high enough when compared with the literature where no deaths were reported. However, it is possible, and indeed likely, that the presence and sub-lethal effects of the measured compounds and elements may have contributed in an additive (or even synergistic) fashion to the effects of the stressor(s) that precipitated the mortalities. The KNP eggs were of course from crocodiles that survived the 2008 and 2009 winter mortalities and might therefore be individuals not at all or less exposed to the lethal stressor(s). The observation that the dead crocodiles were mainly the larger males also point to the possibility that females may eliminate the chemicals via eggs thereby reducing body burden, a process not available to the males. It would therefore be illuminating to analyze and compare the same tissues from male and female crocodiles.

Continued vigilance through chemical monitoring is required to predict and possibly prevent future mortalities. This study has shown that chemical pollutants are present in the top freshwater carnivore of Africa. Other, yet unknown or unsuspected, pollutants may contribute towards the threats, but the compounds and elements that were considered here require continuous attention.

7. Recommendations

- Because Ca and other inorganic elements are extracted from the shell during embryo development, it is recommended that the elemental profile of the egg content and eggshells at the time of egg deposition and afterwards be determined and followed. It is also important to investigate the possible ability of the adult females to depurate certain inorganic elements such as Hg through eggs.
- The origin and pollutant sources of the chickens fed to the farmed crocodiles should be determined.
- The concentration data of the organic pollutants in individual eggs would also further support determining which effects and which compounds may be associated with eggshell thinning, if at all.
- The KNP natural reference site (Nhlanganini Dam) might have been too close to the Olifants and Letaba rivers. It is recommended that other or more reference sites be used in the future.
- Future studies should explore the possible associations of inorganic and organic pollutants with eggshell thickness. Possible confounders, such as age of the crocodile, distribution of eggshell thickness per clutch, and condition of the female, need to be controlled.
- Determining the sex ratios of juvenile and adult crocodile populations in polluted and reference areas may shed more light on possible chemical pollutant impacts. In the absence of accurate historical sex ratio data, the effect of pollution over time might be very difficult to assess. Laboratory experiments may be contemplated whereby eggs are dosed and the combined (synergistic) effect of temperature and endocrine disruption on sex determination be investigated.
- Detailed analysis of preserved tissues from male and female crocodiles might shed more light on the dynamics of pollutants in different sexes due to transfer of pollutants (and therefore dilution of the pollutants in the body of the females) to eggs.

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